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SCIENTIFIC EDITION

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CONTENTS OF THIS ISSUE

Possible Complex Formation Between Macromolecules and Certain Pharmaceuticals X. The Interaction of Some Phenolic Compounds with Polyethylene Glycols, Polypropylene Glycols, and Polyvinylpyrrolidone 659
David Guttman and *Takeru Higuchi*

The Hypotensive Action of 1-Phenyl-2-Dimethylaminoethoxymethyl Tetrazole Hydrochloride (TT-209) 664
G. K. W. Yim, E. G. Gross, and *H. H. Keasling*

A Study of the Carbon-14 Content of Blood After the Oral Administration of C^{14}-Carboxyl Acetylsalicylic Acid to Non-fevered and Fevered Rats 667
Ross E. Crabtree, John B. Data, and *John E. Christian*

Anticonvulsant Properties of Some Alkyldiols, Alkyldiones and Related Compounds 669
Duane G. Wenzel and *Gilbert Y. Koff*

The Assay of Aminoacetic Acid Elixir by Nonaqueous Titration 672
Gordon Meland and *M. I. Blake*

The Effect of Sodium N-Lauroyl Sarcosinate and of Sodium Dehydroacetate on the Enzymes of the Gastrointestinal Tract 675
Charles W. Bauer and *Edward Francis La Sala*

Selective Media for Study of the Bacterial Flora of the Mouth Following Use of Oral Antiseptics 678
Nancy E. Watson and *George F. Reddish*

The Detection of Glutethimide (Doriden®) and a Metabolite in Dog Urine 681
Herbert Sheppard, Barbara S. D'Asaro, and *Albert J. Plummer*

The Evaluation of Various Emulsifiers Including Polyoxyethylene Sorbitan Monostearate (Tween 60) for Parenteral Fat Emulsions 685
G. Frederick Lambert, Jonathan P. Miller, and *Douglas V. Frost*

The Comparative Anatomy of Certain Species of *Smilax* 691
James D. Caponetti and *Maynard W. Quimby*

A General Study of the Effects of Lipids upon the Quantity of Peroxides Produced by Photosensitized Zinc Oxide 696
Frederick Claire Blubaugh and *Earl P. Guth*

Metal Chelates of Alloxan 699
Winthrop E. Lange and *William O. Foye*

Alkaloids of *Chelidonium majus*, L., Leaves and Stems I. *dl*-Tetrahydrocoptisine 702
F. J. Bandelin and *W. Malesh*

An "o-Hydroquinone" Analog of Thyroxine 704
H. R. Wetherell

The Assay of *Rauwolfia serpentina* Preparations 708
Daniel Banes, Jacob Wolff, Herman O. Fallscheer, and *Jonas Carol*

The Oxidative Degradation of Reserpine Preparations 710
Daniel Banes, Jacob Wolff, Herman O. Fallscheer, and *Jonas Carol*

Book Notices 712

Second N. F. X Interim Revision Announcement V

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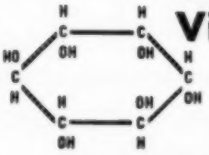

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The next annual meeting of the American Pharmaceutical Association will be held in New York, N. Y., April 28 through May 3, 1957. Titles of papers must be submitted to the appropriate Section secretary not later than January 15, 1957 and abstracts (not to exceed 200 words) not later than February 15, 1957, or preferably earlier, in order to assure a place on any Section program.

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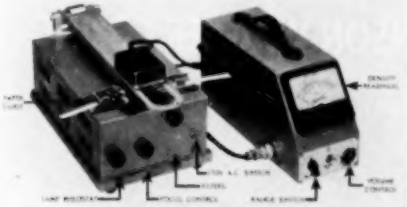

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SECOND INTERIM REVISION ANNOUNCEMENT

NATIONAL FORMULARY X

Official from October 1, 1956

By virtue of the authority conveyed in Chapter III, Article X and Chapter IX, Article V of the By-Laws of the American Pharmaceutical Association, the Council of the Association appointed the Committee on National Formulary, approved the text of this Interim Revision Announcement prepared by the Committee, authorized the printing thereof, and fixed October 1, 1956, as the date upon which it shall become official.

ROBERT P. FISCHELIS, *Secretary*
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Committee on National Formulary

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ADDITIONS, CHANGES, AND CORRECTIONS

The page numbers cited, unless otherwise indicated, refer to N. F. X.

Calcium Lactate, page 122—Change the first sentence in the paragraph entitled *Magnesium and alkali salts* to read:

Magnesium and alkali salts—Mix 1 Gm. of Calcium Lactate with 40 ml. of water, carefully add 1 ml. of hydrochloric acid, heat the solution to boiling, and add rapidly 40 ml. of oxalic acid T.S.

Cobalamin Concentrate, page 167—Delete the paragraph entitled *Microbiological assay* and replace it by the following:

Assay—Proceed as directed under *Cobalamin Assay*, page 3, This Interim Revision Announcement.

Dihydrocodeinone Bitartrate, page 193—Change *Identification Test B* to read:

Identification—
B: Dissolve about 250 mg. of Dihydrocodeinone Bitartrate and 500 mg. of hydroxylamine hydrochloride in 2.5 ml. of water contained in a 50-ml. Erlenmeyer flask. Cover the neck of the flask and heat on a steam bath for 35 minutes. Cool to room temperature and add ammonia T.S., dropwise, until the oxime precipitates, shaking the flask after the addition of each few drops of ammonia T.S. Place the flask in an ice bath for 10 minutes, and collect the oxime on a small suction filter. Wash the precipitate with 5 ml. of water containing 1 per cent ammonia (made by diluting 1 ml. of ammonia T.S. to 10 ml.), remove the excess water by suction, and dry the precipitate for 3 hours at 105°: the dihydrocodeinone oxime melts with decomposition between 261° and 265°, page 691.

Hexavitamin Capsules, page 271—In the first paragraph following "... 2 mg. of thiamine hydrochloride," add "or an equivalent amount of thiamine mononitrate, ..."

Hexavitamin Tablets, page 273—In the first paragraph following "... 2 mg. of thiamine hydrochloride," add "or an equivalent amount of thiamine mononitrate, ..."

Ichthammol, page 294—Change the first line of the paragraph entitled *Loss on drying* to read:

Loss on drying—Dry Ichthammol at 80° for 8 hours and continue. ...

Iodine Solution, page 299—Change the first four lines in the paragraph entitled *Assay* to read:

Assay—Place exactly 5 ml. of Iodine Solution in a 500-ml. glass-stoppered flask, and dilute with 10 ml. of water. Titrate with 0.1 *N* potassium arsenite using starch T.S. as the indicator. Each ml. of 0.1 *N* potassium arsenite is equivalent to 12.69 mg. of I. To the titration mixture add 25 ml. of hydrochloric acid and 5 ml. of. . . .

Iodine Tincture, Strong, page 300—Change the first four lines in the paragraph entitled *Assay* to read as directed under *Iodine Solution*.

Lithium Bromide, page 330—In the paragraph entitled *Chloride* change "(700 parts per million)" to "(0.7 per cent)."

Mannitol, page 347—Change the paragraph entitled *Melting range* to read:

Melting Range—Mannitol melts between 165° and 168°, page 691.

Methionine, page 377—Change the first paragraph in the monograph to read:

Methionine contains not less than 99 per cent of $C_5H_{11}NO_2S$.

Change the paragraphs entitled *Ammonium salts*, *Heavy metals*, *Iron*, and *Assay* to read:

Ammonium salts—

Stock solution of ammonium chloride—Dissolve 78.5 mg. of ammonium chloride in sufficient water to make exactly 250 ml., and dilute 10 ml. of this solution to 100 ml. The dilute solution contains the equivalent of 10 mcg. of NH_3 in each ml.

Procedure—Place 5 Gm. of sodium hydroxide pellets and 300 ml. of water in a 500-ml. distillation flask. Remove ammonia from the solution by distilling until 25 ml. of the distillate gives no color with 0.5 ml. of alkaline mercuric-potassium iodide T.S. Allow the solution in the distillation flask to cool and add 200 mg. of Methionine. Distil, collecting two 25-ml. fractions in 50-ml. Nessler tubes. Add 0.5 ml. of alkaline mercuric-potassium iodide T.S. to the distillates and to a series of standards, prepared by dilution of the ammonium chloride stock solution, containing the equivalent of 0, 5, 10, 15, and 20 mcg. of ammonia in 25 ml. of solution. Stopper the tubes and invert them several times to mix their contents. After allowing 10 minutes for color development, determine the amount of ammonia present in the distillates by comparing their color intensities with those of the standards. If the second 25 ml. of distillate from the Methionine sample contains appreciable ammonia, distil and collect additional 25-ml. fractions and determine their ammonia content. Methionine contains not more than 100 parts per million of NH_3.

Heavy metals—Dissolve 500 mg. of Methionine in 20 ml. of water, add 1 ml. of 1 *N* hydrochloric acid and water to make 25 ml.: the heavy metals limit, page 678, for Methionine is 20 parts per million.

Iron—Dissolve 1 Gm. of Methionine in 40 ml. of water, add 2 ml. of hydrochloric acid and about 50 mg. of ammonium persulfate, dilute to 50 ml., and mix well. To this solution add 3 ml. of ammonium thiocyanate T.S., and mix thoroughly: the color obtained is no darker than that produced in 50 ml. of a solution containing 70 mcg. of ferrous ammonium sulfate (equivalent to 10 mcg. of Fe) when treated in the same manner. Methionine contains not more than 10 parts per million of Fe.

Assay—Dissolve 12 Gm. of sodium acetate and 2 Gm. of potassium iodide in 100 ml. of water in a glass-stoppered flask. Determine the pH of the solution, and, if necessary, adjust it with 0.1 *N* hydrochloric acid to a pH of between 6 and 7. Add about 300 mg. of Methionine, accurately weighed, to the solution and shake the mixture until the Methionine is dissolved. Add exactly 50 ml. of 0.1 *N* iodine, ...

Petrolatum, Liquid, Light, page 431—Change the second sentence of the first paragraph to read:

Not more than 10 parts per million of a suitable form of tocopherol may be added as a stabilizing agent.

Pyrogallol, page 478—Change the paragraph entitled *Melting range* to read:

Melting range—Pyrogallol melts between 130° and 134°, page 691.

Rutin, page 498—Change the Category statement, page 500, to read:

CATEGORY—Bioflavonoid.

Sodium Thiocyanate, page 547—Change the first two lines in the paragraph entitled *Arsenic*, page 548, to read:

Arsenic—Sodium Thiocyanate meets the test for *Arsenic*, page 649. Prepare the solution to be tested (*under a well-ventilated hood*) as follows:

GENERAL TESTS, PROCESSES, AND APPARATUS

Cobalamin Assay—Add the following procedure on page 659, preceding the section on *Congealing Range or Temperature*.

COBALAMIN ASSAY

Cyanocobalamin Tracer Reagent—Dilute an accurately measured volume of a standardized solution of radioactive cyanocobalamin * with sufficient water containing 1 per cent of benzyl alcohol to yield a solution having a radioactivity between 500 and 5000 counts per minute per ml. Determine the radioactivity of this reagent by measurement in a suitable counting assembly, adjusting the concentration of the tracer reagent so as to obtain optimum counting accuracy with the particular assembly used. Store in a refrigerator.

Standardization—Prepare a solution in water of a weighed quantity of U. S. P. Cyanocobalamin Reference Standard to contain between 20 mcg. and 40 mcg. in each ml. To determine the degree of recovery of a known amount of cyanocobalamin, perform the entire assay on a 10-ml. portion of this solution, proceeding as directed under *Assay Preparation*, beginning with "Add water to make a measured volume . . ." Calculate the recovery factor F by the formula $(50 \times A_{361})/(0.207 \times E \times R)$, in which R is the quantity, in mcg. of U. S. P. Cyanocobalamin Reference Standard in the portion of the solution taken, and E is defined under *Calculation*.

Cresol-Carbon Tetrachloride Solution—Mix equal volumes of carbon tetrachloride and freshly distilled cresol.

Phosphate-Cyanide Solution—Dissolve 100 mg. of potassium cyanide in 1000 ml. of a saturated solution of dibasic sodium phosphate, and mix well.

Butanol-Benzalkonium Chloride Solution—Mix 1 volume of a 12.8 per cent solution of benzalkonium chloride with 9 volumes of normal butyl alcohol.

Alumina-Resin Column—Place a pledget of glass wool in the bottom of a constricted glass tube such as a 50-ml. buret. With the tube held in an upright position, add a volume of a slurry of ion-exchange resin in water sufficient to give a column of settled resin 7 cm. in height. When the solid has settled somewhat, allow the water to drain so that there is only 1 cm. of liquid above the resin column, and tamp the resin lightly. Then add a volume of a slurry of alumina in water sufficient to increase the height of the settled column to 10 cm., and allow the water to drain to about 1 cm. from the top of the alumina. Add a pledget of glass wool, and wash the column, using a total of 50 ml. of water and again draining to within 1 cm. of the top of the column. Prepare a fresh column for each determination.

Assay Preparation—Transfer to a beaker a weighed quantity or measured volume of the preparation to be assayed, equivalent in vitamin B_{12} activity to that of 200 to 500 mcg. of cyanocobalamin. Add water to make a measured volume of not less than 25 ml., then add 5.0 ml. of *Cyanocobalamin Tracer Reagent*. Add, while working *under a hood*, 5 mg. of sodium nitrite and 2 mg. of potassium cyanide for each ml. of the resulting solution. Adjust the solution to approximately pH 4 with diluted hydrochloric acid, and heat on a steam bath for 15 minutes. Cool, and adjust the pH to between 7.6 and 8.0 with sodium hydroxide T.S. Centrifuge or filter to remove any undissolved solids.

Procedure—Transfer the *Assay Preparation* to a 250-ml. centrifuge bottle, add 10

* A solution of cyanocobalamin made radioactive by the incorporation of Co^{60} is available from Merck and Co., Inc., Rahway, N. J. Orders must be accompanied by authorization Form 374 after approval by the United States Atomic Energy Commission, Oak Ridge, Tenn.

ml. of *Cresol-Carbon Tetrachloride Solution*, suitably close the bottle with a glass, polyethylene, or foil-wrapped rubber stopper, shake vigorously for 2 to 5 minutes, and centrifuge. Remove and save the lower, solvent layer. Repeat the extraction using a 5-ml. portion of *Cresol-Carbon Tetrachloride Solution*, and combine the lower, solvent-layer extracts in a centrifuge bottle or separator of 50- to 100-ml. capacity.

Wash the combined extracts with successive, 10-ml. portions of dilute sulfuric acid (1 in 7) until the last washing is practically colorless (two washings usually suffice). During each washing, shake for 2 to 5 minutes, allow the layers to separate, centrifuge if necessary, and discard the acid layer. Wash further with two successive, 10-ml. portions of *Phosphate-Cyanide Solution*. Finally, wash with 10 ml. of water. Discard all of the washings.

To the washed extract add 30 ml. of a mixture of 2 volumes of *Butanol-Benzalkonium Chloride Solution* and 1 volume of carbon tetrachloride. Extract with two successive, 5-ml. portions of water, each time shaking vigorously for 1 minute, centrifuging, and removing and saving the upper, aqueous layer.

Pass the combined aqueous extracts through the *Alumina-Resin Column* at a rate of about 1 ml. per minute, maintaining a 1-cm. layer of liquid on the head of the column by adding water as needed. Discard as much of the forerun as is colorless (usually about 5 ml.), and collect the colored eluate (usually about 10 ml.) in a 50-ml. centrifuge tube or separator containing 0.5 ml. of diluted acetic acid. Extract the eluate by shaking for 2 to 5 minutes with 5 ml. of *Cresol-Carbon Tetrachloride Solution*, and discard the upper, aqueous layer. To the extract add 5.0 ml. of water, 5 ml. of carbon tetrachloride and 10 ml. of normal butyl alcohol. Shake, allow to separate until the upper layer is clear, and remove the upper, aqueous layer.

Determine the absorbance of the aqueous extract at 361 mμ and 550 mμ in a spectrophotometer suitable for measurements in the ultraviolet region, using a tungsten light source. Make the 361-mμ reading using a filter capable of reducing stray light. Calculate the ratio A_{361}/A_{550}: the purity of the aqueous extract is acceptable if the ratio is not less than 3.10 and not more than 3.40. If a ratio outside this range is observed, purify the aqueous extract by repeating the extraction cycle, proceeding as directed in the foregoing paragraph, beginning with "Pass the combined aqueous extracts through the *Alumina-Resin Column*," and using a fresh column.

If an acceptable absorbance ratio is observed in the aqueous extract, transfer 1.0 ml. of it to each of 3 suitable aluminum or stainless steel planchets, and evaporate to dryness under an infrared heater. Add 1.0 ml. of *Cyanocobalamin Tracer Reagent* to each of 3 similar planchets, and evaporate to dryness in the same manner.

Measure the radioactivity of the contents of each planchet in a suitable counter over a period optimum for the particular counting assembly used. Average the results, and correct the average for the observed background radioactivity determined over two or more 30-minute periods.

Calculation—Calculate the efficiency, E, of the extraction process by the formula $E = (C_e - C_b)/(C_R - C_b)$, in which C_e and C_R are the average radioactivity counts per minute for the preparation under assay and the *Cyanocobalamin Tracer Reagent*, respectively, and C_b is the average count per minute of the background. The cobalamin content, expressed in mcg. of cyanocobalamin, of the portion taken for assay is given by the formula $(50 \times A_{361})/(0.207 \times E \times F)$, in which F is the recovery factor calculated in the standardization of the *Cyanocobalamin Tracer Reagent*.

Containers—Glass, page 660—Delete the sixth and seventh sentences under *Powdered Glass Test* (in lines 7–13, page 661), and substitute therefor the following:

Shake the sieves for a short time, then remove the glass from the No. 20 and No. 40 sieves, and recrush and sieve as before. Repeat again this crushing and sieving operation. Empty the receiving pan, reassemble the nest of sieves, and shake on a mechanical sieve shaker for 5 minutes or by hand for an equivalent length of time.

Delete the last complete sentence on page 661, and substitute therefor the following:

Reduce the heat so that the autoclave cools at the rate of 0.5° per minute and comes to atmospheric pressure in 38 to 46 minutes, being vented as needed to prevent the formation of a vacuum.

In the section, *Water Attack at 121°*, page 662, delete the fourth sentence beginning in line 7 and ending in line 10 of the paragraph, and substitute therefor the following:

Using a graduated cylinder, transfer 100 ml. of the water from each container or, in

the case of smaller containers, the combined contents of several containers, into a 250-ml. Erlenmeyer flask of resistant glass, add 5 drops of *Methyl Red Solution* and titrate, while warm, with 0.02 *N* sulfuric acid.

In the section, *Acid Attack at 121°*, page 662, delete the third sentence beginning in line 3 and ending in line 6 of the paragraph, and substitute therefor the following:

Fill each container to 90 per cent of its overflow capacity with 0.0002 *N* sulfuric acid in place of water, unless it is expected that more than the equivalent of 0.80 ml. of 0.02 *N* sulfuric acid per 100 ml. of contents will be neutralized by alkaline material extracted from the bottle, in which case use 0.0005 *N* sulfuric acid Cover and autoclave the containers as directed under *Water Attack at 121°*. Remove the containers from the autoclave, and cool to room temperature. Using a pipet, transfer exactly 100 ml. of the acid from each container or, in the case of smaller containers, the combined contents of several containers, into a 250-ml. Erlenmeyer flask of resistant glass, and add 5 drops of *Methyl Red Solution*.

Sterility Tests, page 719—Change the third line of the fourth paragraph under *Culturing*, page 723, to read:

. . . on the third, fourth, or fifth day, suitable portions of this turbid medium to additional tubes of . . . .

REAGENTS AND TEST SOLUTIONS

Reagents, page 741—Reagent specifications for *Alumina* and *Ion-exchange Resin*, required in the *Cobalamin Assay*, page 3, This Interim Revision Announcement, are added to this section.

Alumina—Add the following to page 742:

Alumina (*Aluminum Oxide Acid-washed for Chromatography, Chromatographic Alumina*)—A white or practically white powder. Store it in well-closed containers.

Adsorptive power—

Stock o-Nitroaniline Solution—Weigh accurately 100 mg. of *o*-nitroaniline, and transfer it to a 50-ml. volumetric flask. Add about 30 ml. of benzene, and when solution is effected add benzene to volume, then mix well. Transfer 5.0 ml. of the solution to a 100-ml. volumetric flask, add benzene to volume, and mix well.

Standard o-Nitroaniline Solution—Pipet exactly 10 ml. of *Stock o-Nitroaniline Solution* into a 100-ml. volumetric flask, add benzene to volume, and mix well.

Procedure—Weigh quickly about 2 Gm. of alumina to within ±5 mg. into a dry, glass-stoppered cylinder or test tube. Add exactly 20 ml. of *Stock o-Nitroaniline Solution*, shake vigorously for 3 minutes, and allow to settle. Transfer exactly 20 ml. of the clear, supernatant liquid to a 100-ml. volumetric flask, add benzene to volume, and mix well. Measure the absorbance of this solution and of the *Standard o-Nitroaniline Solution* at 395 mμ in a suitable electrophotometer, using benzene as a blank. Calculate the quantity, in mg., of *o*-nitroaniline adsorbed by the formula $1 - (B + A)$, in which A is the absorbance of the *Standard o-Nitroaniline Solution* and B is the absorbance of the solution treated with alumina. Not less than 0.3 mg. of *o*-nitroaniline is adsorbed.

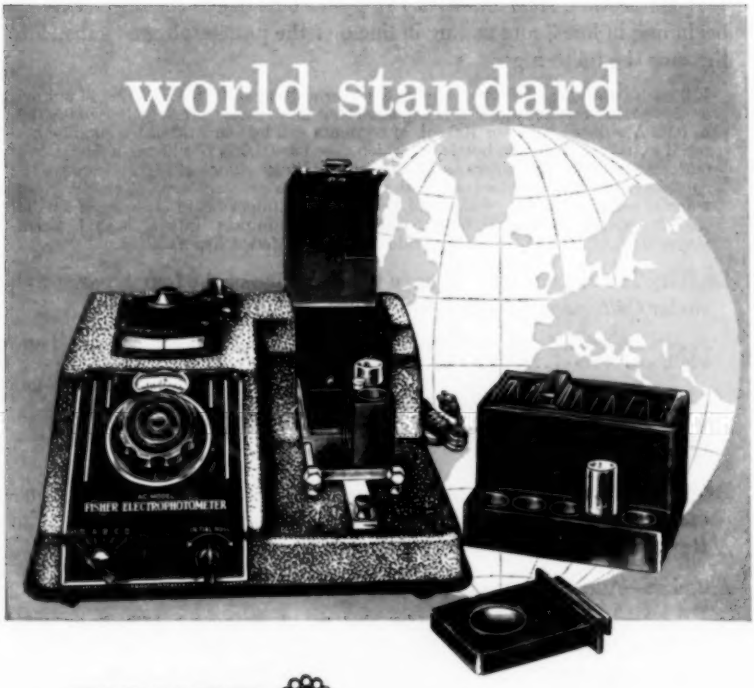
pH—To 5 Gm. in a glass-stoppered, 125-ml. flask add 50 ml. of water, and shake well for 5 minutes. Allow to settle, decant the supernatant liquid, and determine its pH, preferably potentiometrically: the pH is between 3.0 and 5.0.

Loss on drying—Dry about 1 Gm., accurately weighed, at 110° for 3 hours: it loses not more than 5 per cent of its weight.

Ion-exchange Resin—Add the following to page 763:

Ion-exchange Resin*—An intimate mixture of 4 parts of a strongly acidic cation exchanger in the hydrogen form (produced by sulfonation of a styrene-divinylbenzene co-polymer, representing 8 to 10 per cent divinylbenzene) and 6 parts of a strongly basic anion exchanger in the hydroxyl form (produced by amination with trimethylamine of a chloromethylated styrene-divinylbenzene co-polymer, representing 3 to 5 per cent divinylbenzene).

* A suitable resin is that known as "Amberlite MB-1," produced by Rohm and Haas Co., Philadelphia, Pa., and available in small lots from certain laboratory supply firms.



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Possible Complex Formation Between Macromolecules and Certain Pharmaceuticals X*

The Interaction of Some Phenolic Compounds with Polyethylene Glycols, Polypropylene Glycols, and Polyvinylpyrrolidone

By DAVID GUTTMAN and TAKERU HIGUCHI

A photometric-titrimetric method and a dialytic method have been employed to obtain information as to the occurrence of complexes in several systems. Interactions of phenol with members of the polyethylene glycol series, with members of the polypropylene glycol series, and with polyvinylpyrrolidone have been studied. The influence on the complexing tendency of reactant concentration, of salt concentration in the system, of alcohol concentration in the system, and of temperature, has been investigated. The results show that the several systems behave essentially in the same manner despite the differences in the structures of the polymeric substances.

POLYMERIC SUBSTANCES are used frequently in modern pharmaceutical systems. Although the polymers usually used are generally considered to be chemically inert, many of them appear to undergo interactions with small molecules in aqueous solution. These interactions may be classified broadly into two general groups: (*a*) those which do not result in visible physical changes in the systems under study and are, therefore, usually overlooked in the general practice of pharmacy, and (*b*) those which result in phase separations and thus manifest themselves in pharmaceutical systems as rather embarrassing incompatibilities. The first group of interactions is exemplified by the behavior of polyvinylpyrrolidone (PVP) with a number of small molecules including carboxy substituted phenols, phenobarbital, and chloramphenicol (1, 2). Examples of the second type of interaction are, polyethylene glycol (PEG) with iodine in the presence of iodide ions (3) and PEG with phenobarbital (4). These interactions are not particularly peculiar to the polymeric substances since they closely resemble the complexing tendencies of caffeine (5) and other substituted xanthines (6).

In the present investigation both a photometric titrimetric method and a dialytic method have been employed to obtain information as to the behavior of several systems. This study is an extension of the earlier work of Higuchi and Lach (4). Interactions of phenol, with members of the PEG series, with members of the polypro-

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This paper is based on a thesis submitted by David Guttman to the Graduate School, University of Wisconsin, in partial fulfillment of the requirements for the degree of Master of Science.

pylene glycol (PPG) series, and with polyvinylpyrrolidone have been studied. The influences of reactant concentration salt concentration in the system, alcohol concentration in the system, and temperature, were studied. The results show that the several systems studied behave essentially in the same manner despite the differences in the structures of the polymeric substances.

EXPERIMENTAL

The nature and behavior of the interactions between some water-soluble macromolecules and phenolic compounds were determined by studying the conditions under which solutions of the reactants produce systems of varying turbidities. The concentration of phenol, for example, necessary to initiate the separation of phase from a solution of phenol and a polymer was determined by a photometric titration technique. Type titration curves are shown in Fig. 1. It can be seen from these

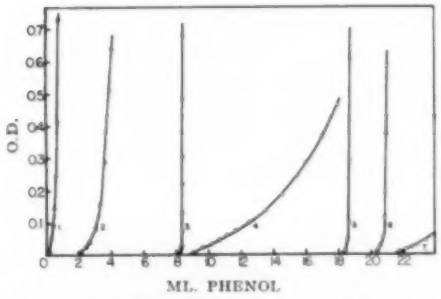

Fig. 1.—Photometric titration curves of several polymers in aqueous solution with 0.55 *M* phenol: 1, polypropylene glycol 1200; 2, polypropylene glycol 750; 3, polyvinylpyrrolidone; 4, polypropylene glycol 400; 5, polyethylene glycol 6000; 6, polyethylene glycol 4000; 7, polyethylene glycol 1500.

curves that the addition of phenol solution to an aqueous solution of the polymer causes no increase in the apparent optical density of the system until a critical concentration of phenol is obtained. At this concentration separation of the complex phase as an oil occurs, the apparent optical density rises sharply and further addition of phenol results in a further increase in the optical density. Extrapolation of the curve, obtained by plotting apparent optical density as a function of ml. of phenol solution added, to zero optical density gives the volume of phenol solution required to initiate separation of the complex. No attempt was made to analyze the shape of these curves. In fact, it was noted that the shape of a curve might vary considerably but the extrapolated end point remained the same within experimental error. Knowing the volume of standard phenol solution required to initiate separation of the complex phase and the initial volume of standard polymer solution, the concentration of reactants necessary to initiate separation can be calculated. From these data, phase diagrams may be constructed as illustrated in Figs. 2 and 3. The experimental points represent the minimum concentrations of reactants necessary to cause the separation of a complex phase. The concentration of the polymeric species has been expressed in terms of equivalents per liter. The macromolecular substances are compatible with all phenol concentrations to the left of a given curve and form a homogeneous one-phase system. A heterogeneous system consisting of the oily complex dispersed throughout the aqueous phase exists to the right of any given curve. The slope and position of these curves qualitatively

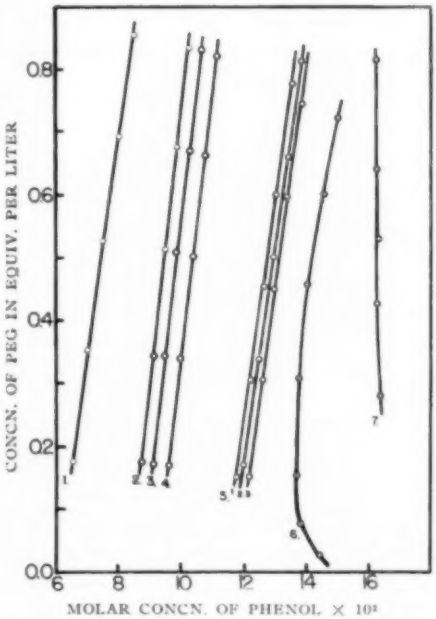

Fig. 2.—Phase diagram showing the interaction of phenol with various polyethylene glycols in water: 1, 2, 3, 4, PEG in the presence of 1 *M* NaCl, 0.5 *M* NaCl, 0.5 *M* KCl, 0.5 *M* LiCl, all at 30°; $5_{1,2,3}$, PEG 6000 at 35°, 30°, 25°; 6, PEG 4000 at 30°; 7, PEG 1500 at 30°.

reflect the equilibrium situations required to initiate separation of the complexes. Results obtained by this method of investigation lead only to qualitative conclusions. The results, however, do provide a method of comparing the reactivities of various water-soluble polymers with phenol and do provide a qualitative insight into the nature of the interactions. With PVP as the exception, other methods of investigation such as dialytic or solubility studies which would yield more significant quantitative data were ruled out by the low molecular weight of the polymers or by the oily indefinite nature of the products formed.

Reactivity of Various Polymers Toward Phenol.—The phase diagrams indicate that similar characteristics are exhibited by all of the macromolecular

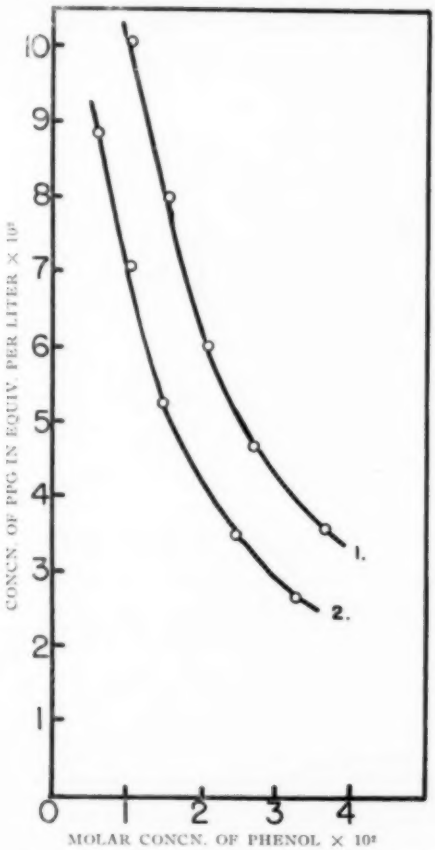

Fig. 3.—Phase diagram showing the interaction of phenol and polypropylene glycol 1200 in water: 1, 27°; 2, 30°.

substances investigated by the photometric titration technique. The most significant characteristic of these interactions is the apparent pronounced dependency on the concentration of phenol and the independency on the concentration of polymer. A similar behavior was noted by Higuchi and Lach (4) in the phenobarbital-PEG system and by Guttman and Higuchi (3) in the I_2-PEG system. The rationalization used by them may be applied to these systems by considering the many sites available for the binding of phenol on a single polymer chain. The interaction is undoubtedly complex and it seems obvious that a single mass law expression would be inadequate to describe the overall interaction. A qualitative expression, however, can be postulated from the interaction, nPhenol + $(CH_2OCH_2)_i$ = $(CH_2OCH_2)_i$:nPhenol; K = $(CH_2OCH_2)_i$:nPhenol/(Phenol)$^n(CH_2OCH_2)_i$.

Although the value of n has not been established, it appears to be of a value sufficient to make the interaction almost totally dependent on the phenol concentration except at very low polymer concentrations. In view of the apparent independency of the interactions on the polymer concentrations, the following decreasing order of reactivity of different polymers toward phenol is apparent from Fig. 1: PPG 1200, PPG 750, PVP, PPG 400, PEG 6000, PEG 4000, PEG 1500.

The sensitivity of the cloud point titration, even with quite dilute polymer solutions, makes it an excellent qualitative test for the presence of these polymers in solution. The inverse relationship between chain length and the concentration of phenol required to initiate separation of the complex phase indicates that a titration of this nature might be of value in differentiating molecular weight members of a polymer group.

The Effect of Neutral Salts on the Phenol-PEG 6000 System.—The addition of neutral salts to this system has a marked effect on the concentration of phenol required to cause the separation of the complex phase as can be seen in Fig. 2, curves 1, 2, 3, and 4. The presence of salts in the system is shown to induce separation of the complex phase at much lower phenol concentrations, the lowering of the phenol concentration being proportional to the salt concentration.

This must be due in part to a dehydrating effect. The nonionic hydrophilic polymers are thought to be solubilized by the association of water molecules to electron donor groups. A simplified mass law expression may be derived from the interaction: nH$_2$O + $(CH_2OCH_2)_i$ = $(CH_2OCH_2)_i n$H$_2$O; K = $(CH_2OCH_2)n$H$_2$O/(H$_2$O)$^n(CH_2OCH_2)_i$. The pronounced dependency of the solvation equilibrium on the concentration, or more correctly the activity, of water is thus apparent. Addition of neutral salts to the system apparently decreases the activity of water and in this manner favors the competing phenol-polymer interaction.

The Effect of Temperature on the Phenol-PEG System and the Phenol-PPG System.—The phenol-PEG interaction exhibits only a minor temperature dependency as can be seen from Fig. 2, curves 5_1, 5_2, 5_3. The phenol-PPG interaction, in contrast, exhibits a marked sensitivity to temperature changes (Fig. 3).

Thermal energy would be expected to exert two antagonistic tendencies on complex formation. It might be expected that these interactions would be favored by thermal desolvation of the polymer chain, thus providing a more suitable environment for the competing interaction with phenol. Thermal agitation might also be expected to result in a decrease in the association of phenol with the polymers by weakening the attractive forces between them. These two tendencies apparently cancel each other in the phenol-PEG system. The complex between phenol and PPG, in contrast, appears to form much more readily at higher temperatures. This would indicate that PPG solvates are relatively unstable. This is supported also by the low solubility of the higher molecular weight polypropylene glycols (7), their pronounced inverse solubility relationship with temperature (8), and their ability to be salted out of aqueous solution by small concentrations of neutral salts.

The Effect of Alcohol on the Phenol-PEG System.—The addition of alcohol to the phenol-PEG 6000 system decreases the complexing tendency as

is shown in Fig. 4. This must be due in part to the lowering of the activty of phenol and to changes in the solvent system.

Phenol-Polyvinylpyrrolidone Complexes.—The interactions of PVP with a number of phenolic compounds were studied by the photometric titration technique. The results of this study (Fig. 5) indicate that the reactivity of PVP toward phenolic compounds simulate those of PEG and PPG.

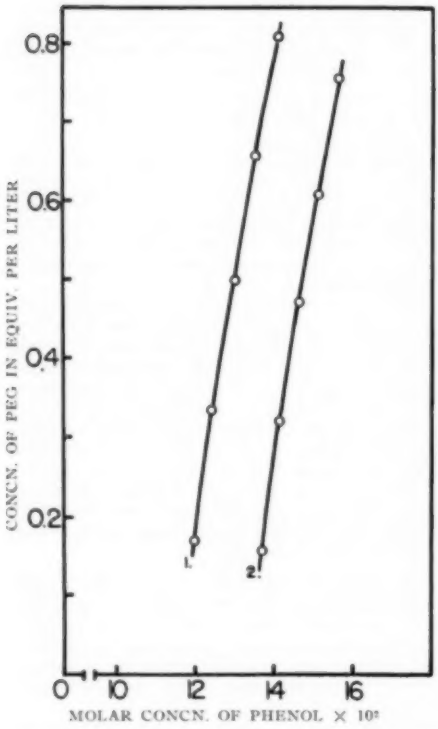

Fig. 4.—Phase diagram showing the interaction of phenol and PEG 6000 in water at 30°: 1, water only; 2, in the presence of 8% ethyl alcohol

PVP, by virtue of its high molecular weight, is suited to dialysis studies such as those described by Higuchi and Kuramoto (2). This procedure is based on the principle that when a solution of a macromolecule, contained with a semipermeable membrane, is immersed in a solution of small molecules, the small molecules bound by the macromolecular component will remain on that side of the membrane containing the macromolecules. The small molecules not bound by the polymer will distribute themselves between the solutions on both sides of the membrane. It was expected that studies of this nature would provide a more quantitative insight into the nature of the interaction.

The results of such a study are shown in Fig. 6. Here the concentration of phenol remaining free in the system is plotted as a function of the phenol

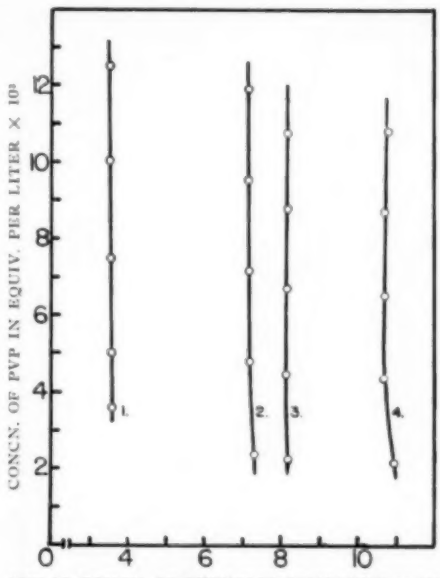

Fig. 5.—Phase diagram showing the interaction of PVP with several phenolic compounds in water at 30°: 1, resorcinol; 2, hydroquinone; 3, phenol; 4, catechol.

concentration initially in the system. If no binding were to occur, this plot would theoretically result in the broken line of Fig. 6., the concentration of free phenol equaling the total concentration of phenol in the system. It is seen, however, from the experimental points of Fig. 6 that an increase in

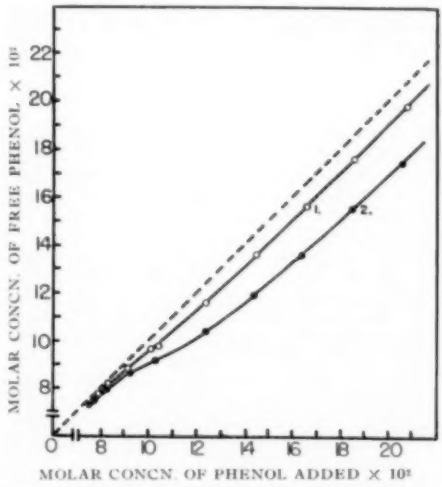

Fig. 6.—A plot showing the interaction of phenol and PVP in water at 0°: 1, initial PVP concn. = 10.5×10^{-3} equiv. liter $^{-1}$; 2, initial PVP concn. = 33.0×10^{-3} equiv. liter $^{-1}$.

the total concentration of phenol in the system results in a decrease in the concentration of free phenol in the system. It was observed that at point R, precipitation of an amorphous complex occurred on that side of the membrane containing the macromolecule. It is seen that after this critical concentration has been reached the amount of phenol bound and effectively removed from the system increases sharply. The upper portion of the curve is seen to run parallel to the theoretical curve. This indicates the binding of a constant amount of phenol and suggests complete precipitation and saturation of the PVP originally in solution. The overall stoichiometry of the interaction can be determined from any point in the upper region of the curve and may be exemplified by the following calculation: Phenol added to the system, 20.0×10^{-3} moles/L.; Phenol free in the system, 16.8×10^{-3} moles/L.; Bound phenol, 3.2×10^{-3} moles/L.; Initial concentration of PVP, 3.3×10^{-2} equiv./L. Bound phenol in moles liter^{-1}/Equiv. of PVP liter^{-1} = 0.94. The stoichiometry of the interaction is thus found to be approximately one phenol molecule bound to every vinylpyrrolidone repeating unit.

These studies have shown that at low phenol concentrations the binding tendency is comparatively small. At a critical concentration precipitation occurs with an abrupt increase in the binding tendency. Although the experimental evidence is inconclusive it is reasonable to assume that the sharp break in the curve at a critical concentration of phenol indicates some configurational change in the polymer chain. The water-soluble polymers under study are thought to exist in solution as partially convoluted linear chains. It may be that at the critical point, precipitation occurs due to the collapse of the essentially linear chain into a more compressed configuration. This rearrangement, apparently initiated by the binding of relatively few phenol molecules, possibly makes available many more binding sites for phenol attachment since this change of state is accompanied by a significant increase in phenol binding.

It is interesting to note the similarity between the phenol-PVP interaction and the behavior of phenol with a number of proteins. Cooper and his co-workers (9, 10) have reported that phenol and cresol are distributed between water and proteins in the emulsoid state according to a partition law. At a certain phenol concentration the protein is precipitated and this change of state is accompanied by an increase in the solvent power for phenol. The similarity of this system to the PVP-phenol system is striking.

Phenolic compounds in general appear to interact with the three polymer types investigated. In the majority of cases, visible signs of complex formation in the form of dispersible oils or solid precipitates are observed. Table 1 summarizes the results of qualitative test-tube studies. The positive sign indicates formation of a precipitate or the separation of an oil when aqueous solutions of the reactants were mixed. It should be noted that although in some cases no visible signs of complexing were noted, formation of soluble complexes probably did occur.

TABLE 1

Phenolic Compound	PVP, K = 30	PEG, 6000	PPG, 1200
Phenol	+	+	+
Resorcinol	+	+	+
Catechol	+	+	+
Hydroquinone	+	+	+
Tannic acid	+	+	+
Pyrogallol	+	+	+
Benzoic acid	−	−	+
p-Hydroxybenzoic acid	−	−	+
Salicylic acid	−	−	+
Sodium salicylate	−	−	−
Methylparaben	−	−	+
β-Naphthol	+	+	+
Picric acid	−	−	−

Reagents.—Redistilled reagent grade phenol; aqueous solutions of polyethylene glycol 4000[1], polyethylene glycol 1500, polyethylene glycol 6000, PVP, (K = 30), polypropylene glycol 1200[2], polypropylene glycol 750, polypropylene glycol 400; reagent grade sodium chloride, lithium chloride, potassium chloride; absolute ethanol; U. S. P. grade resorcinol, catechol, hydroquinone, pyrogallol, *β*-naphthol, picric acid, methylparaben, tannic acid, benzoic acid, salicylic acid, sodium salicylate, *p*-hydroxybenzoic acid; Visking sausage casing.

PROCEDURE

Photometric Titration.—Water, circulated by a Precision Scientific Company constant temperature bath, maintained a glass-jacketed titration vessel at constant temperature. A known volume of polymer solution was placed in the vessel and water was added to make a total volume of 50 ml. A beam of light from a 30-watt tungsten lamp was directed through the solution and focused through a 515 green filter of the photoelectric cell of a Densichron optical density recorder. The light source and the Densichron unit were connected to the line current through a voltage regulator to compensate for line voltage fluctuations. The recording indicator was arbitrarily set at an optical density of 0.00. The solution was stirred continually while phenol solution was added in increments from a buret. One minute was allowed to elapse between successive increments. At a critical concentration, separation of the complex occurred resulting in a rise in the apparent optical density as the complex phase was dispersed throughout the system. Apparent optical density was plotted as a function of ml. of phenol solution added. The curve obtained was extrapolated to 0.00 optical density to give the amount of phenol solution necessary to initiate separation of the complex. Calculation of the molar concentration of phenol and the equivalent concentration of polymer was based on the total volume of solution in the titration vessel when separation first occurred.

Salt Studies.—Constant salt molarity was maintained by making all solutions used in titration to the salt molarity required.

[1] Distributed by the Carbon and Carbide Chemicals Corporation under the trade name of Carbowax.

[2] Distributed by the Dow Chemical Corporation under the trade name of Polyglycol.

Alcohol Studies.—Constant alcohol concentrations were maintained by making all solutions used in titration in the required alcohol concentration.

Dialysis Studies.—Varying amounts of phenol solution were bureted into 125-ml. wide-mouth glass-stoppered bottles. Water was added to make a final total volume of 75 ml. in each bottle. Twenty-five milliliters of PVP solution contained in Visking sausage casing, tied at both ends, was immersed in each bottle. To correct for the binding of phenol by the membrane, control bottles were arranged in the same manner differing only in that the PVP solution was replaced by distilled water. The bottles were mechanically agitated at 30° for fifteen hours. The bags were removed from the bottles and discarded. The outside solution in each bottle was analyzed spectrophotometrically for the phenol concentration. All determinations were made on a Beckman model DU spectrophotometer. The molar concentration of phenol in the control bottle corresponded to the total phenol in the system. That in the corresponding bottle containing the PVP represented the free phenol in the system.

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The Hypotensive Action of 1-Phenyl-2-Dimethylaminoethoxymethyl Tetrazole Hydrochloride (TT-209)*

By G. K. W. YIM, E. G. GROSS, and H. H. KEASLING

1-Phenyl-2-dimethylaminoethoxymethyl tetrazole hydrochloride (TT-209) in doses of 5–10 mg./Kg. i.v. in dogs anesthetized with chloralose or pentobarbital produced falls in blood pressure of 60–80% which returned to pre-injection levels only after one-half to two hours. Tachyphylaxis was observed on repeated administration. Epinephrine, acetylcholine, histamine and carotid occlusion responses were not affected. The hypotensive action of TT-209 was unaffected by atropine, diphenhydramine, hexamethonium, vagal section, or decapitation by means of a head clamp. Increased femoral arterial flow rates in both innervated and denervated dog hindlimbs following intra-arterial TT-209 indicated a direct vasodilator effect. In isolated rabbit heart and dog strain-gage-arch preparations, TT-209 depressed the contractile force of the heart. It is concluded that TT-209 is a direct depressant of cardiac and vascular smooth muscle.

PRELIMINARY PHARMACOLOGIC investigations of a series of substituted aminoethoxymethyl tetrazoles revealed a very pronounced hypotensive action in certain derivatives. The structure-activity-relationships and chemistry of these agents with respect to this hypotensive action have been reported by Cosgrove and LaForge (1). The present study represents a more detailed pharmacologic investigation of this hypotensive effect, as exhibited by 1-phenyl-2-dimethylaminoethoxymethyl tetrazole hydrochloride (TT-209) (Fig. 1).

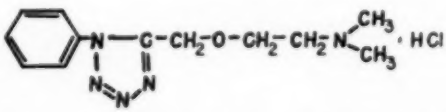

Fig. 1.—1-Phenyl-2-dimethylaminoethoxymethyl tetrazole hydrochloride (TT-209).

EXPERIMENTAL

Methods.—Blood pressure studies were carried out in dogs anesthetized with chloralose, 100 mg./Kg. i. v., or pentobarbital sodium, 36 mg./Kg. i. v. One common carotid or a femoral artery was

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The authors wish to thank Drs. R. O. Hauck and R. A. LaForge for the generous supply of TT-209 and the Bilhuber-Knoll Corporation for a research grant which in part supported this study.

cannulated and blood pressure was recorded by means of a mercury manometer or a transducer. Electrocardiographs were taken with a Sandborn Viso-Cardiette using standard limb leads. Respiratory changes of the anesthetized dogs and also of 3 unanesthetized rabbits were recorded using a respiration recorder similar to that described by Paton (2). Test responses were obtained from epinephrine, acetylcholine, histamine, and carotid occlusion before and after TT-209. The blood pressure effects of TT-209 were also studied in 3 dogs decapitated by means of a head clamp. Ventilation of these dogs was maintained with a Palmer pump.

The direct effect of TT-209 was investigated by injecting 1 to 5 mg. TT-209 into the femoral artery, while the flow rate in the femoral arterial beds was recorded by a rotameter (3). The three-manometer technique of Nolf (4) was employed in studying the comparative effects of the drug on innervated and denervated vascular beds. Changes in heart contractile force were measured with the strain-gage-arch preparation of Walton (5) in dogs artificially ventilated with a Palmer pump. In 3 of the preparations, the stellate ganglia were removed 3–4 weeks prior to the experiment and bilateral cervical vagotomy was performed at the time of the experiment.

The method of Langendorff (6), with slight modifications, was used in studying the effects of TT-209 on the isolated rabbit heart.

compound (1–2.5 mg./Kg.), even when up to two hours elapsed between doses. By slow intravenous infusion, up to 10 mg./Kg. TT-209 was administered without a fall in the systemic arterial pressure. Tachyphylaxis was also observed in animals anesthetized with chloralose.

In 2 dogs which had not previously received TT-209, no drop in blood pressure was observed for one hour following i. m. injections of 20 mg./Kg. of the compound. On the other hand, a small drop of 16–28 mm. (11–22%) was recorded in 3 of 4 dogs four to ten minutes after they were given the same dose by the subcutaneous route. In two unanesthetized dogs, the oral administration of 30 mg./Kg. TT-209 did not produce a hyptotensive response.

TT-209 did not affect the responses to epinephrine, acetylcholine, histamine, or carotid occlusion. Further, the hypotensive response of TT-209 was not blocked by the prior administration of atropine sulfate, diphenhydramine hydrochloride, or hexamethonium chloride.

During initial experiments on dogs anesthetized with chloralose, marked bradycardia and apnea were noted as the blood pressure fell following TT-209. The respiratory effects of TT-209 were then compared with that of Veriloid® in the unanesthetized rabbit. Veriloid in doses of 40 μg./Kg. produced definite slowing of the respiratory rate of the rabbits, however, 10–30 mg./Kg. TT-209 did not alter the respiratory rate. An attempt was

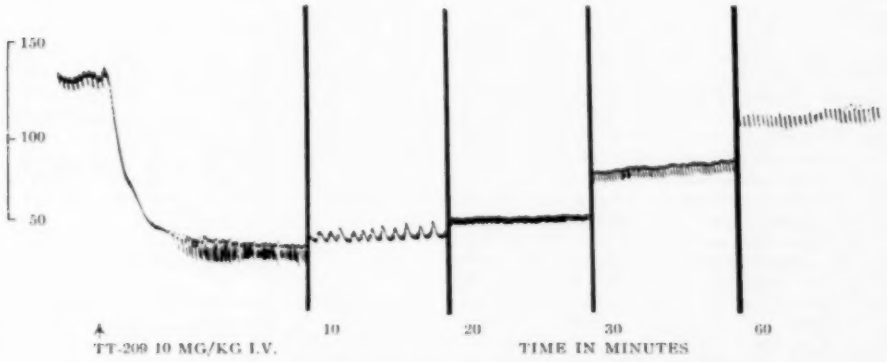

Fig. 2.—Dog carotid blood pressure, pentobarbital anesthesia.

RESULTS AND DISCUSSION

Effects on the Dog Blood Pressure.—TT-209 in doses of 5–10 mg./Kg. i. v. in dogs anesthetized with pentobarbital sodium produced a fall in blood pressure of 60–80%. The blood pressure returned to pre-injection levels after one-half to two hours. Figure 2 illustrates a typical response following 10 mg./Kg. TT-209 i. v. A second i. v. injection of the same dose, administered after the blood pressure had returned to the pre-injection levels, was either ineffective or less effective in lowering the blood pressure. Tachyphylaxis was also observed upon repeated administration of smaller doses of the

then made to evaluate the role of the vagal reflexes in the hypotensive response of TT-209. Ten dogs, anesthetized with chloralose, received i. v. injections of the compound followed in one hour by a dose of 5 mg./Kg. In 5 of these animals, bilateral cervical vagotomy was performed between the 2 doses. As the experiments progressed, it became evident that the alterations in respiration and heart rate that accompanied the hypotensive response of TT-209 were not consistent. Apnea, followed by hyperpnea, or only hyperpnea was seen. Tachycardia, as well as bradycardia was observed. Further, no marked differences were noted in the TT-209 responses in the normal and vagotomized dogs.

Blood pressure responses to TT-209 were also recorded in 3 dogs which were decapitated by means of a head clamp. Ventilation was maintained by a Palmer pump. The hypotensive responses observed were comparable to those obtained in the intact dog, indicating that the higher centers were not critically involved in the hypotensive response to TT-209.

Peripheral Effects of TT-209.—The injection of 1 mg. TT-209 into the femoral artery of 4 dogs was followed by definite increases of the flow rates of the femoral arterial beds, as recorded by a rotameter. No accompanying change was noted in the systemic blood pressure, which was measured from the femoral artery of the opposite hind limb by means of a transducer. Increases in the femoral flow rate also followed higher doses of the compound (2–5 mg.), despite the slight falls produced in systemic blood pressure.

The three-manometer technique of Nolf was employed in order to study the comparative effects of the drug on innervated and denervated vascular beds. The injection of 1 mg. TT-209 into the femoral artery of the denervated hind limb of 4 dogs was followed by a depression of the back arterial pressure of that limb, without any accompanying change in the back arterial pressure of the opposite innervated hind limb or in the systemic arterial pressure. The responses following the intra-arterial administration of the compound to the innervated hind limb were of comparable magnitude to the intra-arterial responses elicited in the denervated hind limb. The back arterial pressure changes of the 2 limbs following the intravenous administration of larger doses of TT-209 were not markedly different. The responses to intra-arterially administered epinephrine were not altered. These results indicate a peripheral vasodilator action of TT-209.

Cardiac Effects of TT-209.—In 3 Langendorff isolated rabbit heart preparations the addition of 1 cc. of 1:1,000 TT-209 to the perfusion fluid was followed by approximately 45% reduction of the amplitude of contraction. Contractions returned to pre-injection amplitude in five minutes. Heart rate was slightly slowed and slight increases or no change in coronary sinus outflow was noted. The positive inotropic responses to epinephrine were not altered. A second dose of 1 cc. 1:1,000 TT-209 was followed by approximately 25% reduction of the amplitude of contraction. Heart rate was slowed from 160 to 78 beats per minute in one instance. Two subsequent doses of TT-209 in one of the hearts elicited depressions of 42% and 52%.

The cardiac effects of TT-209 were further studied in 6 dogs using the strain-gage-arch preparation of Walton (5). In 3 of the 6 dogs, the stellate ganglia were removed 3–4 weeks prior to the experiment and bilateral cervical vagotomy was performed at the time of the experiment. TT-209 in doses of 5–10 mg./Kg. depressed the contractile force of the dog heart by 35–75%. The denervated hearts were similarly depressed by TT-209. No slowing of the heart rate was observed and the positive inotropic responses to epinephrine were not altered.

No changes were noted in the EKG following TT-209, other than the changes in heart rate already mentioned. The effects of TT-209 on ventricular arrythmias, induced by the coronary occlusion procedure described by Harris (7), were also studied. In dogs anesthetized with pentobarbital 25 mg./Kg. and morphine sulfate 1 mg./Kg., 5 mg./Kg. TT-209 i. v. produced the characteristic depressor response, but did not alter the ventricular arrythmias. Similar lack of effect on the arrythmias was noted when TT-209 was administered slowly i. v. so that no fall in systemic blood pressure occurred. In unanesthetized dogs prepared by the Harris procedure, divided doses of TT-209 up to a total of 20–60 mg./Kg. were administered without effect on the ventricular arrythmias. This amount of TT-209 produced convulsive movements or frank convulsions, thus terminating the experiment. The administration of pentobarbital i. v. allayed the convulsions induced by TT-209.

In conclusion, these data suggest that the hypotensive action of TT-209 results from a direct depressant action on cardiac and vascular smooth muscle. Although TT-209 possessed depressant properties similar to those of quinidine, TT-209 was unlike quinidine in that it had no effect on the ventricular arrythmias, and was less effective in lowering the blood pressure upon repeated administration. No evidence of tachyphylaxis to quinidine sulfate was observed when 4–6 doses of 1 mg./Kg. or 5 mg./Kg. were given i. v. at fifteen-minute intervals to 4 dogs. The hypotensive response produced by 5 mg./Kg. quinidine sulfate was of comparable magnitude and duration to the response elicited by 1 mg./Kg. TT-209.

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A Study of the Carbon-14 Content of Blood After the Oral Administration of C^{14}-Carboxyl Acetylsalicylic Acid to Nonfevered and Fevered Rats*

By ROSS E. CRABTREE, JOHN B. DATA, and JOHN E. CHRISTIAN

Salicylates act as antipyretics only on elevated body temperatures. In searching for the mechanism of this action, the blood salicylate levels have been investigated after the administration of an antipyretic dose of aspirin to fevered and nonfevered rats. The concentration of "salicylate" present in the plasma and red cells was determined by use of acetylsalicylic acid labeled in the carboxyl position with carbon-14. Absorption was faster, the peak concentration was greater, and the amount of protein-binding of the salicylate was greater in the fevered rats as compared to nonfevered rats. As the body temperature diminished, the concentration of salicylate in the plasma was greatly decreased.

THERE ARE numerous reports in the literature concerning the blood levels of salicylate after the administration of aspirin. Most of these are concerned with the rate of absorption of aspirin as compared to other analgetics (1). Others deal with the blood levels of salicylate in rheumatic patients following its administration in a series of doses (2).

The work reported here entailed a comparative study of blood levels of salicylate in fevered and nonfevered animals after the administration of a single antipyretic dose of C^{14}-carboxyl acetylsalicylic acid. The object of this research was to gather additional information about the mechanism of the action of aspirin. The radioactivity of the blood samples was determined by comparison with a suitable standard to obtain the concentration of the carbon-14, expressed as salicylate, in the plasma and the red cells.

EXPERIMENTAL

Preliminary Treatment of the Animals.—Adult male rats of the Sprague-Dawley strain weighing between 215 and 280 Gm. were used. Both the nonfevered and fevered animals were fasted for twelve hours prior to the time they were used, but the animals had water at all times.

Fever was produced by the subcutaneous injection of a 15 per cent suspension of Brewer's Yeast (Squibb) in 0.2 per cent sodium carboxymethylcellulose solution. Although in previous work (3) 10 ml./Kg. of a 15 per cent suspension was used, in this study it was found that 1 ml. of suspension per rat produced a satisfactory rise in temperature. An average rectal temperature of 102° F. was reached at the fever plateau which occurred seventeen hours after the injection. Only those rats that reached a fever peak well above average (102.5° F. or greater) were used for the antipyretic experiments.

Antipyretic Dose.—C^{14}-carboxyl acetylsalicylic acid with an activity of 0.45 μc./mg., previously prepared in this laboratory (4), was used. A total aspirin dose of 250 mg./Kg. was administered as a suspension in 1 ml. of freshly prepared solution of 0.4 per cent sodium alginate.[1] This aspirin dose consisted of a mixture of 40 mg./Kg. of the radioactive aspirin, representing an average of 5 μc. per rat, and 210 mg./Kg. of carrier in the form of powdered acetylsalicylic acid, U. S. P. The procedure for oral administration recommended by Comar (5) was used. The dose was introduced from a syringe attached to a catheter and then followed by the administration of 2 ml. of water from an auxiliary syringe.

Blood Levels.—At various time intervals after the administration of a suspension of aspirin, blood samples of approximately 0.3 ml. were taken from the rats by a modification of the orbital sinus method of Stone (6). Two drops of 2 per cent tetracaine hydrochloride were instilled into the eye of the rat ten minutes before samples were taken. A capillary tube was used to puncture the sinus, and the blood was allowed to flow into the tube. As the tube filled, the blood was drawn up into a syringe. Then 0.2 ml. of this blood was diluted with 0.80 ml. of a 0.9 per cent sodium chloride solution containing 0.25 mg./ml. of heparin sodium and the mixture centrifuged. All the supernatant liquid was removed and an aliquot, 0.80 ml., was retained. The proteins in the aliquot were precipitated by the addition of an equal volume of acetone. It was shown by prior experiment that the precipitated protein contained negligible quantities of carbon-14; thus, the activity of the protein-free solutions represented the total activity. The red cells remaining were hemolyzed by addition of 0.25 ml. of water and the proteins precipitated by acetone. After centrifuging, aliquots of each of the protein-free solutions were placed into separate aluminum planchets and dried under infrared heat lamps.

* Received April 13, 1956, from the Bionucleonics Laboratories, School of Pharmacy, Purdue University, Lafayette, Ind.
Presented to the Scientific Section, A. PH. A., Detroit meeting, April, 1956.

[1] Kelcosol, Kelco Company.

The samples were counted in a commercial gas flow counter. Each sample was compared to a reference standard made from an aliquot of the suspension used for administration. The exact nature of the salicylate compounds in the blood following the administration of aspirin is not known; however, since former workers report salicylate to be the predominant substance present, all the carbon-14 levels were calculated as "salicylate." The salicylate levels in both nonfevered and fevered rats are plotted in Fig. 1.

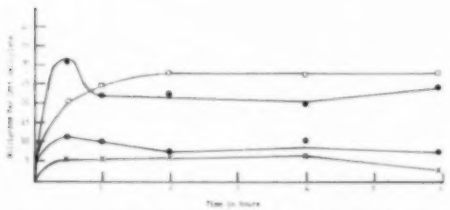

Fig. 1.—Blood levels of salicylate in nonfevered and fevered rats after administration of 250 mg./Kg. of C^{14}-carboxyl acetylsalicylic acid. ●——● Plasma levels of fevered rats; ○——○ plasma levels of nonfevered rats; ⊕——⊕ red cell concentration of nonfevered rats; ×——× red cell concentration of fevered rats. Each point represents the mean value for at least 6 animals. At one-half hour $t = 4.0$ for the plasma levels.

At the same time that the blood samples were taken, the rectal temperatures of the rats were taken. The results of these readings are recorded in Fig. 2. The temperatures of the nonfevered rats did not vary more than about one degree. The elevated temperature of the fevered rats was lowered to normal within two hours and remained normal for the remaining course of the experiment.

Since blood samples taken during periods of fever appeared to be more viscous than normal blood, a determination of the hematocrit values of the blood during the period of antipyresis was performed on 5 rats. The results are plotted in Fig. 3.

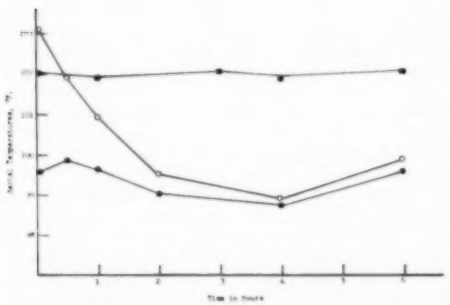

Fig. 2.—Rectal temperatures of rats after administration of 250 mg./Kg. of aspirin. ■——■ fevered rats without aspirin; ○——○ fevered rats administered aspirin; ●——● nonfevered rats administered aspirin. Each point represents the mean value for at least 6 animals.

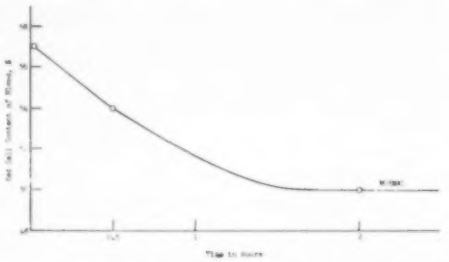

Fig. 3.—The change in hematocrit values of blood of rats undergoing antipyresis. Each point represents the mean value for at least 5 animals.

DISCUSSION

Figure 3 indicates that the ratio of plasma to red cells increased during antipyresis. This is in accord with the beliefs of other workers that salicylates may act by bringing about dilution of the blood (7). According to these findings water shifts from the inner tissues to the blood during fever. The excess water in the blood is then vaporized from the peripheral tissues which causes a cooling effect and lowers the body temperature. In rats the water is probably vaporized chiefly by route of the respiratory tract. This dilution of the plasma no doubt changes the concentration of salicylate and may account in part for the differences shown in fevered and nonfevered animals.

A marked difference in the concentration of salicylate between fevered and nonfevered animals is evident in both the plasma and red cells. The maximum concentration of salicylate in the plasma of the nonfevered rats occurred at about two hours; the maximum concentration of salicylate in the plasma of the fevered rats occurred within one-half hour. The average of the maximum concentrations in the fevered animals was 31 mg. per cent whereas in the nonfevered rats it was 28 mg. per cent. Increased metabolic rates during fever (8) may be one reason for the more rapid absorption of salicylate in the fevered rats.

The effect of the plasma dilution is plainly seen by examination of the data. The initial salicylate concentration in the fevered animals fell rapidly as the fever was assuaged and leveled at about 22 mg. per cent and remained thus for six hours after the dose was administered. After this period the concentration of salicylate in the plasma of the fevered rats was not significantly different from that in the plasma of the nonfevered rats. The significant difference in concentration seems to be at the one-half and one hour periods. In some of the fevered rats there was actually a difference of 10–15 mg. per cent in the concentration of salicylate in the plasma levels at these two periods.

A difference in the concentrations of salicylate in the red cells of fevered and nonfevered rats was also observed. However, the difference was a decrease instead of an increase as found in the plasma levels. Nonfevered rats had a high concentration of salicylate in the red cells at the half-hour period. The concentration in the red cells of fevered rats

at this same period was about half the value for the cells of nonfevered rats and still remained lower than normal after the fever had subsided.

If the concentration of salicylate in the red cells represents the proportion of nonprotein bound salicylate in the extracellular fluids as previously reported (9), then during fever more of the salicylate was protein-bound than normally. This excess binding could be interpreted to mean that there was less salicylate to exert its physiological effect in fevered animals than in nonfevered animals, unless the protein-salicylate complex itself has an antipyretic action.

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Anticonvulsant Properties of Some Alkyldiols, Alkyldiones and Related Compounds*

By DUANE G. WENZEL and GILBERT Y. KOFF

A number of alkyldiols, alkyldiones, related structures and derivatives were tested for their anticonvulsant activity with mice. The alkyldiones were generally inactive and toxic. Anticonvulsant activities and toxicities of the diols could be related to the position of the hydroxyl groups and other structural features. The most active compound for maximal electroshock seizures was 2,5-hexanediol, while for Metrazol® convulsions it was 4-hydroxy-4-methylpentanone-2.

VARIOUS MONO- and polyhydroxy alcohols have been reported to exert protection against experimental chemoshock and electroshock. Driver (1, 2) and Chu, *et al.* (3), first observed this effect with isopropyl alcohol. Ethanediol, 1,2-propanediol, 1,3-butanediol, 1,2,3-propanetriol, and 1,2,4-butanetriol were compared to isopropyl alcohol for effectiveness against both electroshock and chemoshock by Bornmann (4). He concluded that 1,3-butanediol was at least as effective as isopropyl alcohol. The diol, 2,2-diethyl-1,3-propanediol (DEP, Prenderol®) and related derivatives of 1,3-propanediol and 1,2-propanediol have been extensively studied as potential anticonvulsants (5–14). Unsaturated alcohols such as methylparafynol have proved effective (15–17), but appear to be hepatoxic (18). Recently a variety of secondary and tertiary monohydroxy alcohols were found to effectively modify electroshock seizures (18).

The objectives of this study were to determine the optimal positions of the hydroxyl groups of some diols and generally the effect of molecular size and configuration. It was also decided to explore the possibility that the alcohols might be active as the corresponding carbonyl biotransformation products. Most of the useful anticonvulsants contain one or more carbonyl groups and a number of alcohols are known to be degraded in the body through the aldehydes or ketones (19).

With the exception of several diol derivatives which were prepared according to usual chemical procedures, the compounds tested in this study were obtained from commercial sources.[1]

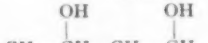
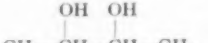
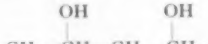
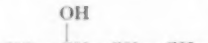
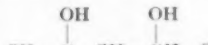
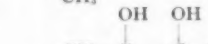
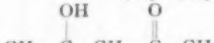
EXPERIMENTAL

Methods.—Albino male mice weighing from 15 to 22 Gm. were used in all experiments. Compounds were tested by oral administration following a fasting period of eight hours. Aqueous solutions of the compounds were used in most cases. Some of the insoluble derivatives were prepared as homogeneous aqueous suspensions with tragacanth.

* Received April 26, 1956, from the School of Pharmacy, University of Kansas, Lawrence.
Abstracted from a portion of the data contained in a thesis submitted to the Graduate School of the University of Kansas by Gilbert Y. Koff in partial fulfillment of the requirements for the degree of Master of Science.

[1] The authors wish to thank the following companies for the compounds supplied: Carbide and Carbon Chemicals Company for 2,3-dimethyl-2,3-butanediol; 1,4-butanedione; 2,4-pentanediol; 2,4-pentanedione; 2,5-hexanediol; 2,5-hexanedione; 2-methylpentanediol-2,4; 4-hydroxy-4-methylpentanone-2, and 2-methyl-2-pentenone-4. Celanese Corporation of America for 1,3-butanediol and 2,3-butanediol; General Aniline and Film Corporation for 1,6-hexanediol, and Jefferson Chemical Company for 4-methyldioxolone-2.

TABLE I.—ANTICONVULSANT ACTIVITY OF DIOLS AND DIONES IN MICE[a]

Name	Formula	LD_{50} mM./Kg.	Maximal Electroshock PD_{50} mM./Kg.[b]	Maximal Electroshock PI[c]	Metrazol Seizures PD_{50} mM./Kg.[b]	Metrazol Seizures PI[c]
1,3-Butanediol		144 (116–179)	39.2 (43.2–56.1)	2.93 (2.29–3.75)	25.8 (20.0–33.3)	5.58 (4.01–7.76)
2,3-Butanediol		110 (94.0–129)	26.8 (24.6–29.2)	4.10 (3.47–4.84)	18.2 (11.4–28.9)	6.04 (3.71–9.85)
2,4-Pentanediol		55.6 (48.3–63.9)	9.76 (8.13–11.7)	5.70 (4.56–7.12)	9.00 (6.67–12.2)	6.18 (4.48–8.53)
2,5-Hexanediol		41.0 (35.3–47.6)	5.04 (3.71–6.85)	8.13 (5.81–11.4)	7.12 (5.05–10.0)	5.76 (3.97–8.35)
2,5-Hexanedione		20.9 (15.7–26.1)	...[d]	...[d]	10.5 (7.66–14.4)	1.99 (1.62–2.51)
2-Methylpentanediol-2,4		26.2 (22.8–30.1)	6.72 (5.84–7.73)	3.90 (3.25–4.32)	3.84 (2.74–5.28)	6.82 (4.77–9.75)
2,3-Dimethylbutanediol-2,3		28.6 (26.7–30.6)	16.4 (14.4–18.7)	1.74 (1.61–1.88)	3.44 (2.96–3.99)	8.31 (7.69–8.97)
4-Hydroxy-4-methylpentanone-2		34.0 (31.2–37.1)	9.44 (7.26–12.3)	3.60 (2.75–4.72)	3.73 (2.80–4.96)	9.12 (6.76–12.3)

[a] See results for additional compounds tested but inactive or toxic.
[b] Determined fifteen minutes after administration of compound.
[c] PI = LD_{50}/PD_{50}.
[d] Inactive.
Values in parentheses represent 95% confidence limits.

Each dosage level was administered to eight mice. Results, expressed as the 50% protective or lethal doses in terms of mM./Kg. were derived by a graphic estimation procedure (20).

Maximal electroshock seizures were produced by a half-wave 60-cycle a. c. of 24 ma. for 0.2 second delivered through ear electrodes. The first shock was administered at fifteen minutes following the test compound and repeated at hourly intervals thereafter until activity was no longer observed. Preliminary determinations indicated that peak activity was reached more quickly with these compounds than usual for anticonvulsants. For this

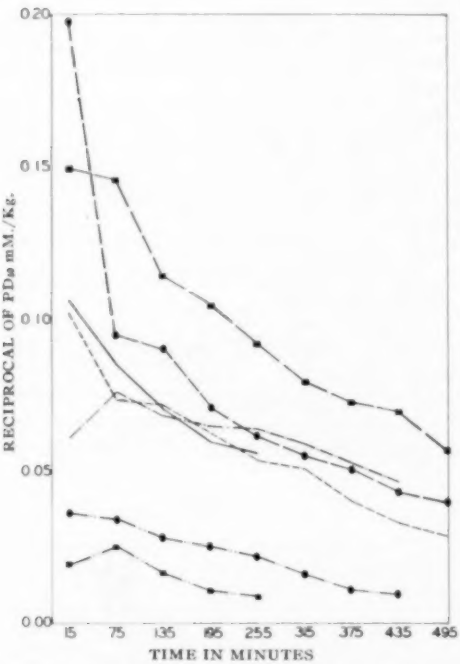


Fig. 1.—Maximal electroshock protection for mice. ■———■ 1,3-butanediol; ●———● 2,3-butanediol; ——— 2,4-pentanediol; ●———● 2,5-hexanediol; ■———■ 2-methylpentanediol-2,4; ——— 2,3-dimethylbutanediol-2,3; ——— 4-hydroxy-4-methylpentanone-2.

reason tests were begun at the fifteen-minute period. If activity was not observed by the seventy-five-minute period it was assumed that the compound was inactive. The criterion of positive protection was abolition of the tonic hindleg extensor phase of the convulsion.

Chemoshock was induced by the subcutaneous administration of Metrazol®, 85 mg./Kg. This was given fifteen minutes following the test compound. Tonic hindleg extension or a clonic spasm lasting for more than five seconds within a one-hour period following the Metrazol were regarded as evidence of lack of protection.

RESULTS AND DISCUSSION

The PD_{50} values obtained with the electroshock and Metrazol tests are given in Table I for the fifteen-minute test period. Figures for electroshock PD_{50} values at the other times tested are not given as all active compounds except 1,3-butanediol and 2,3-dimethylbutanediol-2,3 had peak activity at fifteen minutes. This may be seen from the curves of Fig. 1 in which the reciprocals of the electroshock PD_{50} values are plotted against time. In addition to the compounds listed in Table I, the following were tested but were found to be inactive at nonlethal dosage levels: 1,4-butanediol; 2,3-butanedione; 1,4-butanedione; 2,4-pentanedione; 1,5-pentanediol; 2-methyl-2-pentenone-4; 1,6-hexanediol; 4-methyldioxolone-2 (inner carbonate of 2,3-propanediol); 4,5-dimethyldioxolone-2 (inner carbonate of 2,3-butanediol); β-hydroxybutyric acid, and the bisphenylcarbamates of 2,5-hexanediol, 2,4-pentanediol and 2,3-dimethylbutanediol-2,3.

The diones were found to be considerably more toxic than the corresponding diols and with the exception of the anti-Metrazol activity of 2,5-hexanedione the former were inactive at nontoxic dose levels. The reasons for the lack of anticonvulsant activity by the diones are not obvious as the barbiturates, hydantoins, oxazolidines, and ureides are all di- or triones.

A structure to activity relationship of the diols consistently observed was that compounds with hydroxyl groups on inner carbons were more active and less toxic than when these groups were on terminal carbons. This toxicity and lack of activity of the primary alcohols may be attributed to the fact that when oxidized they can form chemically reactive aldehydes and dicarboxylic acids. The corresponding products of the secondary alcohols would be ketones, while the tertiary alcohols are oxidized only with difficulty (21). The toxicity of the dialdehydes was further evidenced by the fact that 1,4-butanedione was by far the most toxic compound tested. The butanediols were active in the order: 2,3-butanediol > 1,3-butanediol > 1,4-butanediol. The same order of activity related to the position of the hydroxyls obtained with the pentane- and hexanediols.

Although insufficient compounds were available to make any definite conclusions with regard to the effect of the positions of the hydroxyl groups, the three active six-carbon diols have several structural features that appear to influence activity in the same direction. These are: proximity of the hydroxyl groups; whether they are secondary or tertiary diols; and branching of the chain. The figures in Table I indicate that Metrazol activity is improved by decreasing the distance between hydroxyls and at the same time going from a secondary to a tertiary diol with branching. Electroshock activity, on the other hand, was reduced by these same changes. It would be difficult to attribute the differences in activity to any one of these factors in the compounds tested. It is reasonable to assume that as branching occurs steric hindrance would play a part in protecting the functional groups. As previously mentioned, it is also probable that the stability of the tertiary alcohol group is an important factor. The role of the distance between hydroxyls

cannot be assessed from the available information.

Little difference was observed between the activities of 2-methylpentanediol-2,4 and 4-hydroxy-4-methylpentanone-2. The latter is related to the diol by having a carbonyl group in the 2-position rather than a hydroxyl. It would appear desirable to study additional keto-alcohols related to the diols tested.

It has been observed that starvation causes an increased concentration of β-hydroxybutyric acid in the blood (22). The use of starvation as a treatment for epilepsy is well known. The sodium salt of β-hydroxybutyric acid was evaluated in both electroshock and Metrazol tests in order to check the possibility that it is the active agent in starvation and also that it may be an active metabolic product of some of the diols. This compound was found to be inactive as well as nontoxic at doses up to 47.6 mM./Kg.

Inner carbonates of the diols with adjacent carbons and the bisphenylcarbamates of some of the more active diols were prepared. These derivatives not only have structural features that resemble the clinically useful anticonvulsants, but by blocking the hydroxyl groups it was hoped to prolong the activity. None of the derivatives were effective, however, and are listed among the inactive compounds.

SUMMARY

1. Twenty-one alkyldiols, alkyldiones, related structures and derivatives were tested for their ability to modify maximal electroshock and Metrazol seizures of mice.

2. Eight compounds were found to have anticonvulsant activity. Six of these were diols. None of the derivatives were effective. While the diones, with one relatively inactive exception, were ineffective, a keto-alcohol showed activity.

3. The relationship of structure to activity is discussed.

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The Assay of Aminoacetic Acid Elixir by Nonaqueous Titration*

By GORDON MELAND and M. I. BLAKE†

Aminoacetic Acid Elixir, N. F., is assayed by titration in glacial acetic acid using perchloric acid as the titrant. The method is compared with the official procedure. It is rapid, accurate, and has good precision. It is applicable both visually and potentiometrically.

THE MONOGRAPH for aminoacetic acid elixir was described by Green (1) and made official for the first time in N. F. VIII. The aminoacetic acid content is determined visually by the formol titration, a procedure commonly employed for the estimation of amino acids. Several difficulties encountered in the procedure prompted a search for a more suitable analysis of the elixir. The handling of charcoal as an adsorbent proved troublesome and a possible source of error. A

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second difficulty is the observation of a satisfactory and reproducible end point.

Aminoacetic acid, N. F. VIII, in contrast to the elixir, was analyzed for nitrogen content by the Kjeldahl method. Goldstein (2) showed the formol titration to be more satisfactory for the estimation of aminoacetic acid content. This procedure was adopted by N. F. X. The titration is accomplished potentiometrically using a calomel and glass electrode system. The method is simple and rapid

For the past decade titrations in nonaqueous solvents have in many instances replaced the more conventional procedures. Often, they are applicable where other methods fail. Excellent reviews on this subject are available (3–5).

Nadeau and Branchen (6) satisfactorily titrated amino acids as bases in glacial acetic acid with perchloric acid as titrant and crystal violet as indicator. Good precision was reported.

The application of nonaqueous titrations to the analysis of pharmaceuticals appears to be particularly promising. It is essential that interfering substances be excluded or suitable extractive procedures be employed. Techniques which can be applied to a variety of pharmaceutical preparations are described by Pifer, Wollish, and Schmall (3).

This paper reports a simple, rapid, and accurate method for the analysis of aminoacetic acid elixir based on a nonaqueous titration procedure.

EXPERIMENTAL

Aminoacetic acid elixir[1] was assayed by the N. F. X procedure and by titration in glacial acetic acid using perchloric acid as the titrant. The elixir contained a labeled amount of 13.15 Gm. of aminoacetic acid in each 100 ml. The methods are compared on the basis of ten determinations by each procedure.

N. F. X Assay.—Ten samples of aminoacetic acid elixir were assayed as described in N. F. X. The results are shown in Table 1.

The sodium hydroxide solution used in this assay was standardized against reference standard potassium acid phthalate. The average of three determinations was taken as the normality of the solution. The precision of the results was better than 1 part per 1,000. The average per cent recovery of aminoacetic acid, 96.67%, is based on the labeled amount of 13.15 Gm./100 ml. The low results are in agreement with those of Green (1) who employed the same method and reported values of 95.28% and 95.72%. Several sources of error may account for the low results. First, the treatment with 2 Gm. of activated charcoal is a difficult and awkward operation. Although necessary to clarify the solution, it is possible that the charcoal adsorbs sufficient aminoacetic acid to affect the results. The determination of the end point is a second source of error. It was observed that a comparatively wide range in color could be taken for end point comparison in the three titrations required by the assay. The precision and accuracy of the data indicate that the method requires improvement.

TABLE I.—DETERMINATION OF AMINOACETIC ACID BY N. F. X METHOD

Sample No.	Amount Recovered Gm./100 ml.	Recovery Based on Labeled Amount, %
1	12.45	94.68
2	12.88	97.95
3	12.65	96.20
4	12.63	96.04
5	12.61	95.89
6	12.54	95.36
7	12.54	95.36
8	12.94	98.40
9	12.94	98.40
10	12.94	98.40
	Average	96.67
	Standard Dev.	1.38

Nonaqueous Procedure.—Tenth-normal perchloric acid was prepared by adding 8.4 ml. of 72% perchloric acid to about 400 ml. glacial acetic acid in a 1-liter volumetric flask. This was followed by the addition of 20 ml. acetic anhydride. The solution was diluted to the mark with glacial acetic acid and allowed to stand overnight. The exact normality of the perchloric acid solution was determined by standardization against reference standard potassium acid phthalate. The average of three

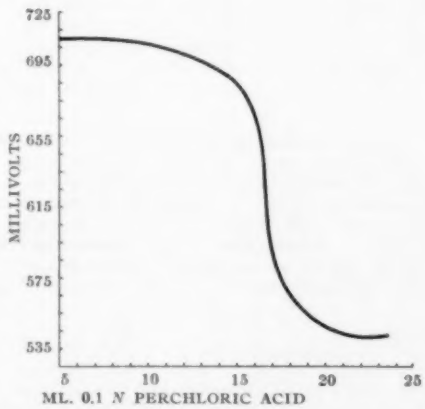

Fig. 1.—Typical titration curve for aminoacetic acid in the N. F. X elixir.

[1] The authors express their appreciation to Eli Lilly and Company for graciously supplying the aminoacetic acid elixir used in this study.

determinations was taken as the normality. The precision was better than 1 part per 1,000

Exactly 1 ml. of aminoacetic acid elixir was transferred by pipet to a 150-ml. beaker. The beaker was heated on a Glas-Col mantle at a temperature of 70–80° until all the liquid was driven off. This required ten to fifteen minutes. Higher temperatures caused the residue to char. While still warm, the beaker was placed on a magnetic stirrer. The stirring bar was placed in the beaker and approximately 50 ml. glacial acetic acid was added. The mixture was stirred (two to three minutes) until solution was effected. The solution, magnetically stirred, was titrated with 0.1 *N* perchloric acid using the Fisher titrimeter and a calomel and glass electrode system. Increments of 0.1 ml. of titrant were added in the vicinity of the end point. Each titration consumed between 16.00 and 17.00 ml. of perchloric acid solution. Figure 1 shows a typical titration curve. The end point is indicated by the sharp break in the curve. The exact end point was determined by plotting $\Delta E/\Delta V$ *versus* V (ml.). The results for ten determinations are shown in Table II.

TABLE II.—DETERMINATION OF AMINOACETIC ACID BY NONAQUEOUS TITRATION METHOD

Sample No.	Amount Recovered, Gm./100 ml.	Recovery Based on Labeled Amount, %
1	13.18	100.23
2	12.97	98.63
3	13.14	99.92
4	13.03	99.09
5	12.95	98.48
6	13.10	99.62
7	13.03	99.09
8	12.97	98.63
9	12.97	98.63
10	12.97	98.63
	Average	99.10
	Standard Dev.	0.19

Based upon the labeled amount of aminoacetic acid, a recovery of 99.10 ± 0.19% is reported. Accuracy and precision are superior by the nonaqueous procedure. Other ingredients in the formula of the elixir do not interfere with the titration. Exclusive of the graphing and calculations, four assays can be conveniently completed in less than one and one-half hours. The official assay, on the other hand, required three hours for a similar number of analyses.

The feasibility of a visual nonaqueous titration was investigated. Samples of aminoacetic acid elixir were treated as described in the preceding paragraph except for the use of the titrimeter. Prior to the titration several drops of indicator solution, prepared by dissolving 0.2 Gm. of methyl violet in 100 ml. of chlorobenzene, were added. With the approach of the end point a range of color changes was observed from purple to blue to green and finally to yellow as the end point was exceeded. The proper end point color change was determined by using the indicator in a potentiometric titration and comparing the color change with the graphic end point. The appearance of the first permanent green tinge served as a suitable end point. Visual titrations may be satisfactorily accomplished when titrated to this end point. Three drops of indicator solution produced the most discernible color change.

SUMMARY AND CONCLUSIONS

A method is described for the analysis of aminoacetic acid elixir using glacial acetic acid as the solvent and 0.1 *N* perchloric acid in glacial acetic acid as the titrant. The procedure is compared with the N. F. X assay which employs the formol titration. The official assay yields low results. Possible sources of error are discussed. The percentage recovery of aminoacetic acid by nonaqueous titration is 99.10 ± 0.19%. Four determinations can be effected in one and one-half hours exclusive of the actual calculations. The method is applicable both potentiometrically and visually.

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The Effect of Sodium N-Lauroyl Sarcosinate and of Sodium Dehydroacetate on the Enzymes of the Gastrointestinal Tract*

By CHARLES W. BAUER† and EDWARD FRANCIS LA SALA

The effect of sodium N-lauroyl sarcosinate and of sodium dehydroacetate on the activity of several enzymes was determined. These enzymes were salivary amylase, rennin, pancreatic trypsin, pancreatic lipase, and pancreatic amylase. The effect of LS and of DA dentifrices on the activity of salivary amylase was determined. The effect of salivary amylase activity immediately after brushing the teeth with several dentifrices was determined. The dentifrices included a LS dentifrice, a DA dentifrice, and a plain dentifrice. Also included was a plain mouthwash, a LS mouthwash, and a dry brush. The effect upon salivary amylase activity after brushing the teeth for one week with a LS dentifrice was determined. Some observations about the variability of human saliva are reported.

THE CURRENT advertising for "anti-enzyme" toothpastes states that these agents inhibit acid-producing enzymes; but in that advertising and in the literature, nothing has been reported about the effect of these agents on the physiologically important enzymes of the mouth and of the gastrointestinal tract. If one were to take the term "anti-enzyme" literally, one would expect such a substance to have inhibiting effects upon all enzymes.

The object of this investigation was to determine the effect of two highly advertised "anti-enzymes," sodium N-lauroyl sarcosinate and sodium dehydroacetate, upon the better known enzymes of the gastrointestinal tract.

This investigation makes no attempt to evaluate the usefulness of "anti-enzyme" toothpastes in the prevention of dental caries.

HISTORY

One of the most widely accepted theories of dental caries is that bacteria, normally present in the mouth, produce or contain enzymes which act upon sugars or other carbohydrates that adhere to the teeth. This action results in the formation of acids. These acids in turn react with the enamel of the teeth to produce dental caries.

With this hypothesis as a basis, Fosdick, *et al.* (1), in 1946, set up various criteria for a suitable agent, to be included in a dentifrice, which would effectively reduce the acidity of the mouth and thereby lower the incidence of dental caries. With these criteria in mind they undertook the investigation of a large number of chemical compounds in the hope of finding one or more which would conform to all the criteria set up. Of 381 compounds tested they found only two which were suitable for inclusion in a dentifrice. The two compounds were sodium N-lauroyl sarcosinate and sodium dehydroacetate. These two compounds are said by Fosdick to act by inhibiting the bacterial enzymes, and not by affecting the bacteria themselves.

This last statement is subject to debate; for Wolf (2) states that sodium dehydroacetate has been shown to inhibit the growth of 13 bacteria, 6 molds, and 1 yeast.

Wolf and Westveer (3) state that several substituted pyrones, among them dehydroacetic acid, were bacteriostatic agents and that the bacteriostatic property of the pyrones was apparently not dependent upon a reaction with the thiol group of essential bacterial enzymes. The thiol group is also an important grouping in many, if not all, of the carbohydrases.

Nothing could be found in the literature about the effect of these two compounds which inhibit the production of acids in the mouth on the physiologically important enzymes in the mouth and in the gastrointestinal tract.

EXPERIMENTAL

Effect of Sodium N-lauroyl Sarcosinate and of Sodium Dehydroacetate on Salivary Amylase.—The effect of sodium N-lauroyl sarcosinate and of sodium dehydroacetate on salivary amylase activity was determined by noting the speed of the hydrolysis of starch by saliva, both in the presence and in the absence of these compounds.

One milliliter of unstimulated human saliva was collected and immediately diluted to 50 ml. with recently boiled and cooled distilled water. In every case the collection and dilution of the saliva was made just before its use.

The composition of the digestion mixtures is shown in Table I.

The components of the digestion mixtures were placed in six-inch test tubes. The saliva solution was added last, and then the tubes were inverted several times to mix the contents. The tubes were then placed in a constant temperature bath at 40 ± 1°. One milliliter of a specially diluted iodine test solution was placed in the depression of

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a white spot-plate. At intervals of two minutes one drop of each of the digestion mixtures was added to 1 ml. of the special iodine test solution. The end point was considered to be reached when no color change was produced in the iodine solution. The first series of tests established the approximate digestion times. The tests were then repeated; and about five minutes before the end of digestion the tests were run at intervals of one minute.

TABLE I.—COMPOSITION OF DIGESTION MIXTURES

Solution of arrowroot starch 1%, freshly prepared	2 ml.	2 ml.	2 ml.
Phosphate buffer, *p*H 7.0	2 ml.	2 ml.	2 ml.
Solution of saliva, 2%	2 ml.	2 ml.	2 ml.
Distilled water	1 ml.	..	..
Solution of sodium N-lauroyl sarcosinate, 1%	..	1 ml.	..
Solution of sodium dehydroacetate, 1%	..	..	1 ml.

The specially diluted iodine test solution was prepared by diluting 1 ml. of 0.1 *N* iodine test solution with distilled water to make 125 ml.

The results of these tests are shown in Table II.

TABLE II.—THE EFFECT OF SODIUM N-LAUROYL SARCOSINATE AND OF SODIUM DEHYDROACETATE ON SALIVARY AMYLASE

	Digestion Time, Min Fastest Time	Slowest Time
Control tube	41	55
Sodium N-lauroyl sarcosinate	90[a]	316[a]
Sodium dehydroacetate	41	55

[a] Discontinued.

The results of these tests indicate that sodium dehydroacetate has no inhibiting effect on salivary amylase. The sodium N-lauroyl sarcosinate greatly retards or entirely inhibits the activity of salivary amylase.

The Effect of Sodium N-lauroyl Sarcosinate and of Sodium Dehydroacetate on Rennin.—The effect of these two compounds on rennin activity was determined by noting the speed of coagulation of milk by rennin both in the presence and in the absence of these substances.

The procedure that was followed was that of the assay of Rennin, N. F. IX, with the following modifications: The test was run in triplicate. One milliliter of distilled water was added to the control. One milliliter of a 1% solution of sodium N-lauroyl sarcosinate was added to the milk in the second series, and 1 ml. of a 1% solution of sodium dehydroacetate was added to the third series. The time for the coagulation of the milk in the controls was twenty-four minutes; for the containers with the sodium N-lauroyl sarcosinate, twenty-five minutes; and for the sodium dehydroacetate, twenty-six minutes.

The results of these tests indicate that neither the sodim N-lauroyl sarcosinate nor the sodium dehydroacetate affects the activity of rennin.

The Effect of Sodium N-lauroyl Sarcosinate and of Sodium Dehydroacetate on Pepsin.—The assay for pepsin, N. F. IX, was followed. The results showed a difference of only 0.6 ml. in the volumes of undigested egg albumen, which indicate that there is no effect on the activity of pepsin by either sodium N-lauroyl sarcosinate or sodium dehydroacetate.

The Effect of Sodium N-lauroyl Sarcosinate and of Sodium Dehydroacetate on Pancreatic Trypsin.—An adaptation of Sörenson's formol titration method (4) was used. Titrations were made at intervals of zero, one, seventeen, and forty-four hours. The results indicated that there is no effect upon the activity of pancreatic trypsin by either sodium N-lauroyl sarcosinate or sodium dehydroacetate.

The Effect of Sodium N-lauroyl Sarcosinate and of Sodium Dehydroacetate on Pancreatic Lipase.—The effect of the two compounds upon the activity of pancreatic lipase was determined by noting the amount of fatty acids liberated from olive oil. The method used was that described by Bauer and Wilson (5), with the following modifications: Two milliliters of distilled water was added to flasks A and B, which served as the controls. Two milliliters of a 1% solution of sodium N-lauroyl sarcosinate was added to flasks C and D, and two ml. of a 1% solution of sodium dehydroacetate was added to flasks E and F. The results of the experiments are shown in Table III.

TABLE III.—THE EFFECT OF SODIUM N-LAUROYL SARCOSINATE AND OF SODIUM DEHYDROACETATE ON PANCREATIC LIPASE

	Ml. of 0.1 *N* Alcoholic Potassium Hydroxide to Neutralize Acidity Trial 1		Trial 2	
Control	Flask A	8.4	Flask B	8.8
Sodium N-lauroyl sarcosinate	Flask C	2.8	Flask D	4.1
Sodium dehydroacetate	Flask E	12.6	Flask F	11.8

The results show that sodium N-lauroyl sarcosinate has an inhibiting effect on pancreatic lipase, whereas sodium dehydroacetate has a stimulating effect upon it.

The Effect of Sodium N-lauroyl Sarcosinate and of Sodium Dehydroacetate on Pancreatic Amylase.—The tests were made in a manner similar to that previously described for salivary amylase. Potato starch was used in place of arrowroot starch, and 2 ml. of a 0.1% suspension of pancreatin was used as the source of amylase.

The results of these tests are shown in Table IV.

TABLE IV.—THE EFFECT OF SODIUM N-LAUROYL SARCOSINATE AND OF SODIUM DEHYDROACETATE ON PANCREATIC AMYLASE

	Digestion Time— Trial 1	Trial 2
Control	8 min.	16 min.
Sodium N-lauroyl sarcosinate	22 hr.[a]	19 hr.[a]
Sodium dehydroacetate	8 min.	16 min.

[a] Discontinued.

The results indicate that sodium dehydroacetate has no inhibiting effect on pancreatic amylase. The sodium N-lauroyl sarcosinate inhibits the activity of pancreatic amylase.

A Study of Dentifrices Containing Sodium N-lauroyl Sarcosinate and Sodium Dehydroacetate.—It has been shown that sodium N-lauroyl sarcosinate inhibits the activity of salivary and pancreatic amylases. The question was whether a dentifrice containing this compound would show this effect.

To answer this question a new series of tests were made, which included dentifrices that contained sodium N-lauroyl sarcosinate and sodium dehydroacetate. In one series of tests 1 Gm. of the dentifrice was added to the digestion mixture and was shaken well. This shaking did not give a uniform mixture. In a second series of tests 2 Gm. of the dentifrice was added to 10 ml. of distilled water, was shaken well, and was filtered. Five milliliters of the filtrate was added to the digestion mixture. It was assumed that 5 ml. of the filtrate was equivalent to one gram of dentifrice. This gave a more nearly uniform mixture.

The method of studying the effects of these substances on the activity of salivary amylase has been previously described. The differences in the composition of the digestion mixtures and the digestion times are shown in Table V.

TABLE V.—A COMPARISON OF THE EFFECT OF SODIUM N-LAUROYL SARCOSINATE AND OF SODIUM DEHYDROACETATE FROM VARIOUS SOURCES, ON SALIVARY AMYLASE

Composition	Digestion Time
Control	26 min.
2 ml. of 1% sodium dehydroacetate sol.	28 min.
2 ml. of 1% sodium N-lauroyl sarcosinate sol.	20 hr.[a]
1 Gm. dentifrice containing sodium dehydroacetate	125 min.[a]
1 Gm. dentifrice containing sodium N-lauroyl sarcosinate	125 min.[a]
5 ml. aq. ext. (1 Gm. dentifrice) sodium dehydroacetate	20 hr.[a]
5 ml. aq. ext. (1 Gm. dentifrice) sodium N-lauroyl sarcosinate	20 hr.[a]

[a] Discontinued.

The results indicate that sodium N-lauroyl sarcosinate, incorporated in a dentifrice as well as in solution, inhibits salivary amylase. The dissimilarity in the results obtained with sodium dehydroacetate required further study in order to be explained. It was shown that the dentifrice containing sodium dehydroacetate also contained sodium oxalate, which is known to inhibit salivary amylase. Hence, the inhibiting effect of the dentifrice on the salivary amylase could be caused by he sodium oxalate.

Effect of Dentifrices and Mouthwashes on Salivary Amylase.—Saliva was collected under various conditions and at different intervals of time. First, an unstimulated saliva was collected to serve as the control. Second, one of the following processes was used: The teeth were brushed with a dry brush containing no dentifrice; they were brushed with approximately one inch of toothpaste; or the mouth was rinsed with 2 ml. of mouthwash. In each case the process was carried out for one minute. Then the mouth was rinsed with two portions of water. Immediately after the rinsing, and at intervals of five and thirty minutes, the saliva was again collected and its digestion time on starch was determined.

The processes used were as follows: (*a*) use of a dry brush with no dentifrice, (*b*) use of a toothpaste containing sodium N-lauroyl sarcosinate, (*c*) use of a toothpaste containing sodium dehydroacetate, (*d*) use of a toothpaste containing neither sodium N-lauroyl sarcosinate nor sodium dehydroacetate, (*e*) use of alkaline aromatic solution N. F. IX, and (*f*) use of alkaline aromatic solution containing 2% sodium N-lauroyl sarcosinate.

The effect of these processes on the activity of salivary amylase, after various intervals of time, is shown in Table VI.

TABLE VI.—EFFECT OF THE DIFFERENT PROCESSES ON SALIVARY AMYLASE

	Digestion Time, Min.			
Processes	Before Brushing	Immediately after Brushing	5 Min. after Brushing	30 Min. after Brushing
(*a*)	52	79	63	50
	29	48	38	32
	30	50	41	27
(*b*)	62	20	37	52
	32	31	38	43
	35	15	45	78
	30	14	38	44
	18	17	26	16
(*c*)	27	12	36	29
	27	24	36	29
	31	21	55	35
(*d*)	11	16	13	12
	30	33	43	26
(*e*)	35	70	45	35
(*f*)	17	39	34	25
	25	29	19	23

The results show that in general there is immediate retardation of salivary amylase activity by the use of the dry toothbrush, the plain toothpaste, and the two mouthwashes. This retardation in activity is temporary and may be due to the dilution factor in rinsing the mouth.

There is immediate activation of salivary amylase activity by the toothpastes containing sodium N-lauroyl sarcosinate and sodium dehydroacetate. This activation is only temporary and within five minutes some slight inhibition develops. It is doubtful that the acceleration of activity is due to either the sodium N-lauroyl sarcosinate or the sodium dehydroacetate. It will be observed that the mouthwash containing sodium N-lauroyl sarcosinate did not produce this temporary acceleration of the salivary amylase. It appears that the temporary activation of the salivary amylase by the toothpastes containing these two ingredients is due to traces of the chloride ion which the other agents do not contain. It is well known that the chloride ion is a salivary amylase activator.

The addition of 1 ml. of sodium chloride solution U. S. P. XV to the digestion mixtures we have

used will reduce the digestion time to about one-fourth the time which has been recorded.

Effect on the Activity of Salivary Amylase of Daily Brushing of the Teeth with a Toothpaste Containing Sodium N-lauroyl Sarcosinate.—The average time for starch hydrolysis by saliva was determined by the method previously described. The teeth were then brushed morning and night for one week. At the end of this period the average time for the starch hydrolysis was again determined.

The average digestion time at the beginning of the experiment was twenty-five minutes. At the end of one week the average digestion time was twenty-six minutes.

These results indicate that there is no effect on salivary amylase activity by a toothpaste containing sodium N-lauroyl sarcosinate, which is said to accumulate on the dental plaque (1).

Additional Observations.—During this investigation it was noticed that the saliva collected after meals showed a greater salivary amylase activity than the saliva collected before meals. Salivary amylase activity seemed to be at its lowest level before breakfast. It was thought that the nature of the food ingested, or some of the metabolic derivatives of the food, might produce a hormonal influence on the secretion and the activity of the saliva. The results of this investigation were inconsistent, and no conclusion was reached.

According to Evans (6) the chewing of food is a mechanical stimulation, which causes an increase in saliva secretion with a resulting increase of salivary amylase. Brushing of the teeth with a dry brush did not produce an increase in salivary amylase activity in the experiment above. However, chewing paraffin did give a noticeable increase.

CONCLUSIONS

The use of anti-enzyme toothpastes containing either sodium dehydroacetate or sodium N-lauroyl sarcosinate does not cause any ill effects upon the physiologically important enzymes of the digestive tract.

The effect of these compounds on six of the digestive enzymes investigated is summarized in Table VII.

TABLE VII.—SUMMARY OF THE EFFECT OF SODIUM DEHYDROACETATE AND OF SODIUM N-LAUROYL SARCOSINATE ON SIX OF THE DIGESTIVE ENZYMES

Enzymes	Sodium Dehydroacetate	Sodium N-lauroyl Sarcosinate
Salivary amylase	No effect	Inhibition
Rennin	No effect	No effect
Pepsin	No effect	No effect
Pancreatic amylase	No effect	Inhibition
Pancreatic trypsin	No effect	No effect
Pancreatic lipase	Stimulation	Retardation

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Selective Media for Study of the Bacterial Flora of the Mouth Following Use of Oral Antiseptics*

By NANCY E. WATSON and GEORGE F. REDDISH

The rinse method is the most satisfactory and accurate sampling procedure for use in studies of the mouth flora. Six special culture media are employed for the following purposes: general or "total" counts, aciduric organisms, lactobacilli, streptococci, staphylococci, and yeast counts. The results obtained by means of the rinse sampling method and the special media employed represent the numbers and kinds of bacteria present on mouth surfaces at the time the rinse samplings are obtained. The relative effects of oral antiseptics when used as a mouth rinse can thus be compared and evaluated.

THE BACTERIAL FLORA of the mouth consist of a variety of microorganisms which vary in numbers from day to day and even at different times during a twenty-four hour period. In studying the kinds and numbers of each, certain special culture media must be used for the purpose. Some of these media are selective for certain groups and others for specific microorganisms.

* Received April 13, 1956, from the Lambert-Hudnut Division of Warner-Lambert Pharmaceutical Co., St. Louis, Mo.
Presented to the Scientific Section, A. PH. A., Detroit meeting, April 1956.

In order to determine the germicidal effectiveness of oral antiseptics against these microorganisms under conditions of use, it is necessary to actually count these bacteria before and after use of these antimicrobic agents. A so-called "total" or general count is not sufficient since some mouth bacteria will not grow on general purpose media, and also no differentiation into specific species is possible in media employed for general counts. It is for this reason that special selective media are necessary in order to determine the germicidal activity of oral antiseptics against the various groups of the bacterial flora present in the mouth.

EXPERIMENTAL

Sampling Procedure

It is essential first of all to select a proper procedure for sampling in order to obtain the most accurate estimation of the kinds and numbers of bacteria present in the mouth. Four such methods have been used, two of which are still employed to a certain extent. A brief evaluation of these methods may be of interest and useful in selecting the best procedure for the purpose.

Swab Method.—Because of the inherent inaccuracies in the swab method for securing quantitative bacterial samples for any purpose, especially from the oral cavity, this procedure has long been discredited. Therefore any data secured by the use of the swab method have little significance, at most only qualitative in character. For these reasons studies on the effects of antiseptics used in the oral cavity should not be based on sampling by this means. This method has largely been superceded by other procedures for the reasons given.

Normal (Unstimulated) Saliva.—Since normal saliva contains all varieties of mouth bacteria, dilutions of such saliva may be used, but because of the physical nature of saliva repeated sampling does not produce consistent results. For this and other reasons, the use of unstimulated saliva has generally been discarded in favor of a more accurate procedure.

Stimulated Saliva.—Although frequently employed for the purpose, there are several reasons why stimulated saliva should not be used. The process of stimulating the flow of saliva actually dilutes the normal saliva already present in the mouth. Saliva as it is secreted from the salivary glands is sterile. Therefore, as sterile stimulated saliva enters the mouth the numbers of bacteria in the saliva already present are reduced according to the length of time stimulation is continued. Therefore repeated stimulation of the flow of saliva, as by chewing paraffin, causes a consequent reduction of the numbers of mouth bacteria in the diluted saliva. Because of this continued reduction in the numbers of mouth bacteria per unit volume of stimulated saliva, tests of the effects of the use of antimicrobic agents in the oral cavity result in bacterial counts that are distorted and misleading. The reduced numbers of bacteria following use of oral antiseptics, when determined by this method, will be due to some unknown degree to the dilution with fresh, sterile saliva. The extent to which such reductions in bacterial counts are attributable to the action of the antiseptic as distinguished from the effect of dilution with sterile saliva is not revealed by this method; for this reason stimulated saliva should not be used as a sampling procedure for this purpose.

Rinse Method.—A far more satisfactory and accurate sampling procedure is the rinse method. This involves rinsing of the oral surfaces with a definite amount of sterile saline solution for a specified time period and plating the rinses in suitable culture media. The kinds and numbers of bacteria removed in this way from the mouth surfaces can be accurately determined. When the test procedure is conducted in a uniform manner consistent results are obtained.

While this is not a standard test, the procedure has been widely used for the purpose. The method has been employed in research on the oral flora in several universities and in many independent research laboratories and others. For example, Ostrolenk, *et al.* (1), stated that the mouth rinse technique "..... offers useful information on the degree of antibacterial activity" of antiseptics under practical conditions of use. Also Appleton (2) in discussing the merits of the rinse method of sampling the oral flora concluded: "But we can obtain estimates of the number of bacteria removable in mouth-rinsings after carrying out these measures, and it is probably legitimate to assume that the number of bacteria left in the mouth is a function of the number removable in mouth rinses."

In this connection it is of interest to quote from a paper by Ostrolenk, Meyers, and Mollari (3): "For this work a rinse technique, utilizing physiological saline (0.85%), seemed to be the simplest and easiest to control. The saline solution could be easily sterilized, the volume could be fixed with precision, its time of application could be governed: with "swishing" of the material, all portions of the oral cavity would be reached, most all individuals were equally skilled in its use, and even the most indolent subject would be persuaded to swish vigorously for thirty seconds." Also Shuster and Iannarone (4) used saline rinse for sampling the mouth flora in a study of the effect of tyrothricin on oral bacteria and reported satisfactory results from such use. It may therefore be concluded that the mouth rinse sampling procedure is more suitable and more accurate than other sampling methods and that the results obtained are more significant.

Culture Media

The following media have been proved useful in studies on the effect of oral antiseptics on the bacterial flora of the mouth: NIH Agar (Difco) for general count, Mitis Salivarius agar (Difco) for streptococci, Chapman Stone medium (Difco) for staphylococci, tomato juice agar special (Difco) for aciduric bacteria, Rogosa agar for lactobacilli, and malt extract agar (Difco) for yeasts.

NIH Agar (Difco) is not a selective medium, but it is employed for determining general or so-called "total" bacterial counts, although certain mouth bacteria do not grow on this medium. However, it is useful as a general purpose index medium represent-

ing a cross section, so to speak, of the numbers of the nonfastidious bacteria present. This medium contains Bacto-Casitone, Bacto-Yeast Extract, Bacto-Dextrose, Sodium Chloride, l'Crystine Difco, and Bacto-Agar (*p*H 7.1).

Mitis Salivarius Agar (Difco).—This medium, selective for viridans streptococci, was used successfully by Chapman (5, 6). It contains tryptose, peptone, dextrose, saccharose, dipotassium phosphate, trypan blue, crystal violet, and agar to which tellurite is added after cooling to 45°. The final reaction is *p*H 7.0. The medium is useful for determining quantitatively the numbers of viridans streptococci in saliva and oral rinses.

Chapman Stone Medium (Difco) (7).—This was developed as a selective medium for staphylococci. The medium contains tryptose, yeast extract, mannitol, certain salts, gelatin, and agar, adjusted to *p*H 7.0. Since only staphylococci grow on this medium, quantitative counts of these organisms in saliva and oral rinses can easily be made. While these organisms are not ordinarily numerous in the normal mouth, they are found in large numbers in certain infections of the oral cavity.

Tomato Juice Agar Special (Difco).—As modified by Hadley (8) and recommended by Jay (9, 10), this medium is selective for aciduric organisms. This medium contains tomato juice, peptone, peptonized milk, and agar and is adjusted to *p*H 5.0. Suitable dilutions of mouth rinses plated in this medium give the total numbers of aciduric organisms, but do not differentiate the species present.

Rogosa Agar (11).—A medium devised as selective for lactobacilli only in which quantitative counts of these aciduric and acid-producing bacteria can be made. This medium contains tryptose, yeast extract, glucose, various acids and salts, and agar, and is adjusted to *p*H 5.4. When suitable dilutions of saliva and mouth rinses are plated in this medium only lactobacilli will grow. Different species of lactobacilli, including *L. acidophilus*, *L. casei*, *L. fermenti*, etc., grow on this medium and colony counts of saliva and oral rinses give quantitative numbers of the lactobacilli present.

Malt Extract Agar (12).—This medium is used to determine the presence and numbers of yeasts in the mouth. This medium contains maltose, dextrin, glycerol, peptone, and agar and adjusted to *p*H 4.6. Yeasts grow in platings made from diluted oral rinses and the counts are quantitative.

Test Procedure

The rinse test should be conducted as follows: two hours after breakfast the (human) subject will rinse the mouth with 20 ml. of sterile saline for thirty seconds. This rinse is then expectorated into a sterile test tube and immediately plated in suitable dilutions in appropriate media. After two hours, to allow for re-establishment of the oral flora, the mouth is rinsed with the antiseptic, 20 ml. for thirty seconds, and discarded. At fifteen and thirty minutes, one and two hours afterwards, the mouth is then rinsed with 20 ml. of sterile saline for thirty seconds and the rinses diluted and immediately plated in appropriate media, the plates incubated at 37° for forty-eight hours and counted. The kinds and numbers of bacteria in the control pre-test rinses and in the post-test rinses can then be compared and the per cent changes calculated.

It is, of course, important that proper dilutions of the saline mouth rinses be made which are suitable for each of the media employed, since the numbers of the different kinds of micro-organisms in the mouth vary considerably. Experience has proved that the following dilutions of the mouth rinses will allow for countable plates in each of the media: NIH agar: 1–1,000, 1–10,000, 1–100,000; Mitis salivarius agar: 1–10,000, 1–100,000, 1–1,000,000; Chapman Stone agar: 1–10, 1–100; tomato juice agar: 1–10, 1–100, 1–1,000; Rogosa agar: 1–10, 1–100, 1–1,000; malt extract agar: 1–10, 1–100, 1–1,000.

These are pour plates with the exception of the Mitis salivarius agar and Chapman Stone medium which are streaked plates. In the pour plates 1 ml. of the diluted mouth rinse is placed in a sterile petri dish and 15 ml. of the melted media cooled to 45° is poured, mixed well, and allowed to solidify. The other two media, Mitis salivarius agar and Chapman Stone medium, are first poured, allowed to solidify, and 0.1 ml. of the proper dilution streaked lightly over the surface with a sterile glass rod. Therefore, 0.1 ml. of the original rinse will give a 1–10 dilution on the medium, 0.1 ml. of 1–10 dilution will give a 1–100 dilution of the rinse on the medium, and 0.1 ml. of 1–100 dilution will give a 1–1000, etc. dilution on the medium.

As a control on the antiseptic, another complete test must be conducted on the following day using sterile saline as the test rinse. The test is conducted as described except that 20 ml. of saline is used as the rinse two hours after the control rinse, plated in the different culture media at fifteen and thirty minutes, one and two hours afterwards. The bacterial reductions due to mechanical removal can then be compared with reductions due to the germicidal activity of the test antiseptic.

INTERPRETATION OF RESULTS

Consistent results are obtained when the rinse method of sampling is used and suitable dilutions of the rinses plated in the above culture media. The reason is that a definite quantity of saline rinse is employed for a specified time period and the rinsing process can be duplicated by each individual at different times. Representative samplings of the bacteria present on the surfaces of the mouth are thus obtained.

It must be emphasized that the mouth rinses do not remove all of the bacteria in the mouth. However, the numbers and kinds removed constitute a function of those that were present on oral surfaces at the time of the tests, and also constitute an index of those that remain on these surfaces. The relative effectiveness of oral antiseptics can be evaluated by determining the percentage changes in the kinds and numbers of bacteria present in the mouth following the use of such measures. The numbers of aciduric organisms, the lactobacilli, streptococci, staphylococci, and yeasts, as well as the so-called "total" counts in the mouth rinses must be considered as indexes of the numbers of each present on oral surfaces at the time of each test rinse. When results on these various media

are interpreted in this manner, much valuable information can be obtained in the study of the oral flora and the relative value of various hygienic measures determined.

The results of these tests can best be indicated in terms of percentage reductions. This is done by comparing the bacterial counts from the various hygienic measures determined.

This method has been widely used for the purpose with satisfactory results. Recent reports by Shuster and Iannarone (4) and Florestano, Bahler, and Richards (13) have again demonstrated the value of the method in studies on the effect of oral antiseptics under practical conditions of use. As stated by Florestano, *et al.* (13): "The numbers and types of microorganisms removed from the mouth may then serve as an index of those remaining." This is the purpose of the tests described here and the results obtained are interpreted accordingly.

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The Detection of Glutethimide (Doriden®) and a Metabolite in Dog Urine*

By HERBERT SHEPPARD, BARBARA S. D'ASARO, and ALBERT J. PLUMMER

A method of analysis for glutethimide and a metabolite, α-phenyl glutarimide is described. It is based on the formation of a hydroxamic acid derivative which then complexes with ferric ion to give the purple color. Doriden was administered orally to dogs and the urinary excretion of free and conjugated glutarimide was studied. The free form proved to be α-phenyl glutarimide and the conjugated form was found to yield α-phenyl glutarimide upon acid hydrolysis.

GLUTETHIMIDE (α-phenyl-α-ethyl glutarimide) is a new sedative (1) which is not a barbiturate and has not been found to be habit-forming. In order to obtain some information as to the excretion and metabolism of glutethimide, a colorimetric method has been developed based on the formation of a hydroxamic acid derivative with alkaline hydroxylamine. With the aid of this method the excretion of glutethimide and a metabolite, α-phenyl glutarimide, was studied.

EXPERIMENTAL

In Vivo

Glutethimide was administered orally to mongrel male dogs in a single large dose of 250 mg./Kg. and the urine collected at various time intervals, being certain to empty the bladder by catheterization.

* Received March 29, 1956 from the Research Department, Ciba Pharmaceutical Products Inc., Summit, N. J.
The authors wish to acknowledge the assistance of Doctor Alfred E. Earl and his group for the animal work connected with this study.

Reagents

Hydroxylamine hydrochloride, 2 *M* (store in cold); sodium hydroxide, 3.5 *N;* hydrochloric acid, 3.5 *N;* ferric chloride, 0.37 *M* in 0.1 *N* hydrochloric acid; glutethimide standard solution, 10 micromoles/ml. (217 mg. dissolved in 100 ml. of ethanol); α-phenyl-glutarimide standard solution, 10 micromoles/ml. (189 mg. dissolved in 100 ml. of ethanol); benzene, reagent grade; chloroform, reagent grade; methanol, absolute, reagent grade; aluminum oxide, Woelm, acid-activity I.[1]

Urine Analysis

Free glutarimide.—Extract 25 ml. of urine with an equal volume of benzene by shaking for thirty minutes. Remove the benzene layer and re-extract the urine layer with 15 ml. of benzene. Combine the benzene extracts, concentrate them to about 5 ml. *in vacuo* with the aid of some heat, and dry the residue with anhydrous sodium sulfate. Pour the dried benzene extract and sodium sulfate over an aluminum oxide column prepared as follows: Place 1 gram of Woelm's aluminum oxide previously dried

[1] Obtained from Alupharm Chemicals, 51 C Street, Elmont, Long Island, N. Y.

in vacuo into a 25-ml. buret filled to the top with dried benzene and fixed with a Teflon stopcock and a plug of glass wool above the stopcock. The aluminum oxide is allowed to settle, after which time a plug of glass wool is placed on top. The column is then washed with the benzene contained in the buret. After the sample has been added to the column it is washed with an additional 25 ml. of benzene and the benzene eluates discarded. Twenty-five milliliters of a chloroform-methanol 9:1 mixture dried with sodium sulfate is added to the column and 25 ml. of eluate is collected. The chloroform-methanol eluate is evaporated to dryness and a color reaction performed as for the standard curve. Ninety to 96 per cent recovery of glutethimide added to urine and put through the column was obtained.

Conjugated glutarimide.—To the aqueous phase remaining after the benzene extraction described above add 3.6 ml. of concentrated hydrochloric acid and reflux for six hours, which was shown by preliminary experiments to yield complete hydrolysis of the conjugated material. Cool the solution, extract with benzene and proceed as with free glutarimide. However, approximately five grams of aluminum oxide is used in order to handle the larger amount of solids in this fraction. Here, too, all of the glutarimide should be in the first 25 ml. of the chloroform-methanol eluate.

Paper Chromatography

Aliquots of the eluates from the column are mounted on Whatman No. 1 filter paper and developed with a 1:1 mixture of petroleum ether and methanol. After irrigation of the paper is complete, it is air-dried and sprayed lightly with a 1:1 mixture of hydroxylamine and sodium hydroxide solutions. After thirty minutes the purple color is developed by spraying the paper with 0.37 *M* ferric chloride solution further acidified by the addition of 1 ml. of concentrated hydrochloric acid per 10 ml. of solution. The R_f of gluthethimide is found to be 0.83, while that of α-phenyl glutarimide is 0.67.

Preparation of Standard Curve

Place 0.5 to 5.0 micromoles of glutethimide or α-phenyl glutarimide into 25 ml. of the control urine and proceed as for free glutarimide. After evaporation of the chloroform-methanol solution is complete, add 1 ml. of absolute methanol to dissolve the residue, followed by 1 ml. of hydroxylamine solution and 1 ml. of sodium hydroxide solution in just that order. Mix and let the solution stand at room temperature for thirty minutes. Then add 1 ml. of hydrochloric acid and mix, followed by 1 ml. of ferric chloride reagent. Mix the solution and read within five minutes in the Klett-Summerson colorimeter using filter No. 54.

RESULTS

Since the reaction of imides with hydroxylamine reagent has been mentioned only briefly in the literature (2, 3) it was necessary to work out some of the optimum conditions for this reaction. Figure 1 shows the effect of time and temperature on the formation of the hydroxamic acid derivative of glutethimide. When the reaction was carried out

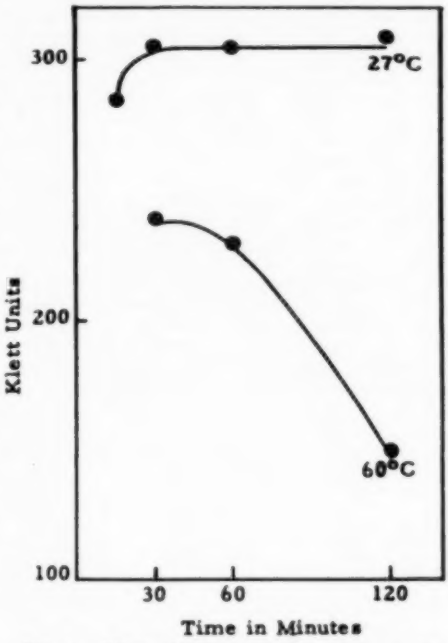


Fig. 1.—Effect of time and temperature on the formation of the hydroxamic acid derivative of glutethimide.

at room temperature, maximum color was obtained in thirty minutes and remained stable over two hours. At 60°, however, the reaction proceeded to a lesser extent and decomposition occurred fairly rapidly. If the reaction was carried out in boiling water, no color was obtained. Subsequent analyses were therefore carried out at room temperature. The concentration of the ferric chloride solution was found to influence the intensity and stability of the color. As can be seen in Fig. 2, the use of 0.74 *M* ferric chloride gave the greatest intensity and stability of color while 0.37 *M* ferric chloride gave a slightly lower intensity of color and this appeared stable only over the first five-minute period. The

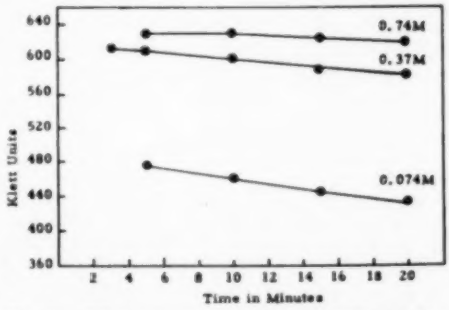


Fig. 2.—Effect of concentration of $FeCl_3$ solution on the intensity and stability of color.

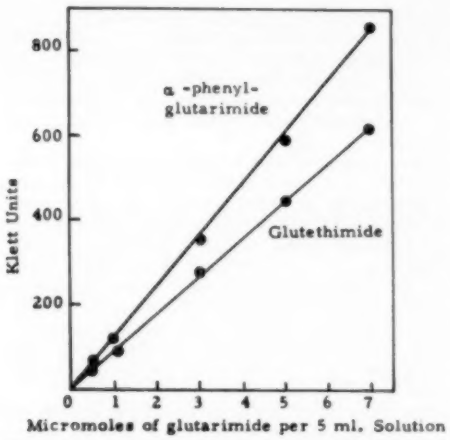


Fig. 3.—Standard curves for gluthethimide and α-phenyl-glutarimide.

0.074 *M* solution of ferric chloride was completely unsatisfactory. In subsequent analyses, however, the 0.37 *M* solution was used because too high a blank was obtained with the 0.74 *M* ferric chloride solution. Figure 3 illustrates the amounts of glutarimide that can be determined by this method, as well as the difference in chromogenic value between α-phenyl α-ethyl glutarimide and α-phenyl glutarimide. The molar ratio of absorbances for α-phenyl glutarimide: α-phenyl-α-ethyl glutarimide is 1.35. The results of the *in vivo* studies can be found in Fig. 4. The blackened columns represent the free glutarimide, calculated as glutethimide, while the white columns represent the conjugated glutarimide, calculated as α-phenyl glutarimide, which was reported to be present by Kebrle and Hoffmann (4). It will be noted that the area of each column represents the total amount of glutethimide excreted during that collection period. Approximately 0.4–1.5 per cent of the administered gluthethimide is excreted in the free form while 3.5–5 per cent is excreted in the conjugated form in twenty-four hours. It will be noted that the greatest rate of excretion of the conjugated form was during the period ending at twenty-four hours after administration of glutethimide, while that of the free form is during the period just beginning at that time. If the free form were actually glutethimide, one would expect it to be excreted at a faster rate than the conjugated form, which is obviously a metabolic product of glutethimide. When the extracts were subjected to paper chromatography, it was discovered that the free glutarimide was actually α-phenyl glutarimide and that no free glutethimide was present. These chromatograms were run with and without added glutethimide to make certain that materials in the extracts were not changing the characteristic R_f values.

DISCUSSION

Chemically the reaction of glutethimide (I) with

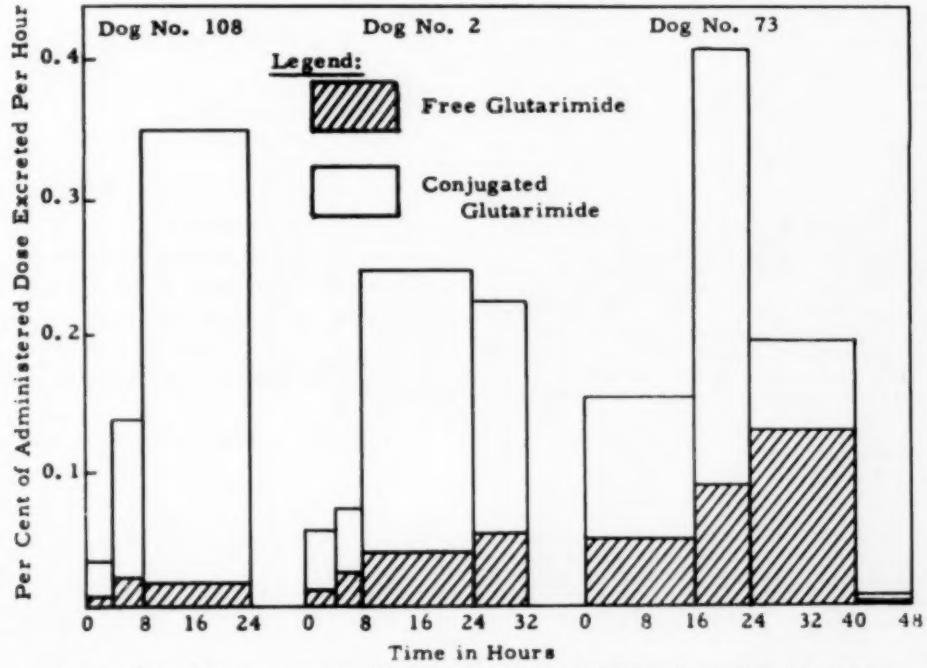
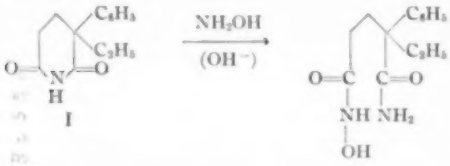


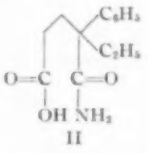
Fig. 4.—Rate of urinary excretion of free and conjugated glutarimide after oral administration.

hydroxylamine reagent probably proceeded as outlined below:



I

It is unlikely that the other C—N bond is cleaved, since that bond is extremely resistant to ordinary alkaline hydrolysis and yields no hydroxamic acid when reacted with hydroxylamine reagent. These reactions were attempted with the hydrolysis product of glutethimide (II).

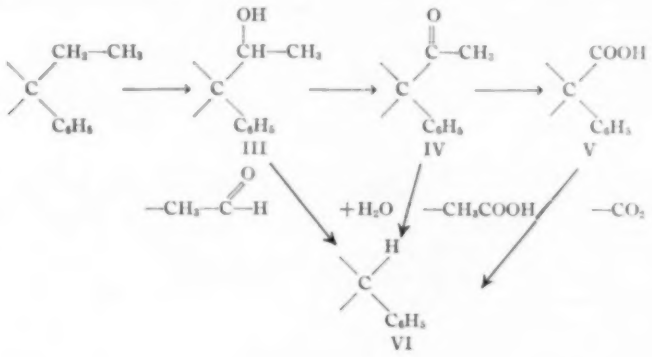


II

The observation that only α-phenyl glutarimide was present in the urine suggests that this route of excretion may not be available to the unmetabolized glutethimide. This has been suggested by studies of Bernhard, *et al.* (5), using C^{14}-labeled glutethimide in which it was demonstrated that the bile was a major route of excretion. The observation that the free α-phenyl glutarimide reaches its maximum rate of excretion later than the conjugated form suggests that the conjugated glutarimide is composed of a precursor of the free form. The exact nature of the conjugated form is still a subject of investigation in several laboratories. Kebrle and Hoffmann (6) recently suggested that oxidation of the ethyl side chain may proceed as follows with the formation of α-phenyl glutarimide (VI) from compound III being favored.

The appearance of free VI in the urine indicates that further oxidation of the ethyl side chain must occur even though to only a small extent in the dog. Bernhard, *et al.* (5), using glutethimide labeled in the ethyl side chain were able to demonstrate the appearance of 1 – 4.5 per cent of the administered C^{14} as $C^{14}O_2$ in rats. This is of the same order of magnitude as the free glutethimide found in the urine of the dog. These same authors using glutethimide labeled with C^{14} in the 2-position showed the appearance of $C^{14}O_2$ indicating further breakdown of the ring structure.

The method of analysis for gluthethimide as described here may be applied to its determination in blood. Whole blood can be satisfactorily extracted with ethyl acetate, the ethyl acetate then evaporated *in vacuo*, and the residue taken up in benzene and put through the column as with the benzene extract for urine. Ninety-six per cent recoveries are obtained by this method.



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The Evaluation of Various Emulsifiers Including Polyoxyethylene Sorbitan Monostearate (Tween 60) for Parenteral Fat Emulsions*

By G. FREDERICK LAMBERT, JONATHAN P. MILLER†, and DOUGLAS V. FROST

Various emulsifying agents were evaluated as regards suitability for use in commercial fat emulsions designed for intravenous therapy. The properties of Tween® 60 were promising enough to warrant a detailed investigation. Animal studies of emulsions stabilized with 0.5 per cent Tween® 60 were encouraging but clinical studies indicated such emulsions to be unsatisfactory for use in the human.

SEVERAL WORKERS have reported on the preparation and clinical use of intravenous fat emulsions (1–13). The efficacy of such emulsions, most of which contain lecithin for emulsification, has become well established through these investigations. Despite the fact that reactions do occur which are not understood, the essential safety of such properly prepared emulsions has appeared encouraging, based on many favorable clinical reports. Although the problem is a major one from the commercial standpoint, very little has been reported on the keeping qualities of intravenous fat emulsions. Emulsions which degrade to yield undesirable products under practical storage conditions would require rigid distribution and careful dating. The inherent instability of lecithin represents a problem which we have attempted recently to define in a preliminary way (14).

In the present study, partial evaluation is made of a large number of emulsifying substances other than lecithin. An early objective was to develop, if possible, an emulsion containing only one emulsifying substance rather than a mixture of such materials. Of the emulsifiers studied, Tween® 60[1] appeared to offer some promise for further work as evaluated by laboratory and animal studies. Limited clinical tests have now shown Tween® 60 fat emulsions to be unsatisfactory for use in humans (15). In the interim Tween® 60 emulsions have been used by several investigators (16–26). Because of the clinical failure of Tween® 60-containing emulsions their preparation has been abandoned.

METHODS

Preparation of Emulsions.—The emulsions were the oil-in-water type containing the emulsifier with 10 or 15% corn, sesame, or coconut oil in pyrogen-free water. Generally the emulsions were made isotonic by 0.9% sodium chloride or 5% U. S. P. dextrose. The components were premixed by high-speed mechanical stirring and then transferred to a Cherry-Burrell Junior Viscolizer[2] through which the emulsion was recycled at 3,000 p. s. i. for thirty minutes.

In early experiments the emulsions were not autoclaved, but were sterilized by homogenization at 90–100°. The combination of heat and shearing action is apparently capable of destroying *B. subtilis* spores. More recently, however, we have been able to successfully autoclave the Tween® 60 emulsions. Most of the emulsions used in these tests were autoclaved.

Mouse Toxicity Test.—An intravenous mouse test was used to screen emulsifying agents for acute toxicity. The emulsifier, if water-soluble, was dissolved at 2% or less in either isotonic saline or dextrose, and the solution injected into the tail vein of three or more mice in the weight range of 18–25 Gm. The injection was made in about eighteen seconds and the animals were observed for at least twelve hours thereafter. If the emulsifier was not water-soluble, it was tested as an oil-in-water emulsion. In no case did we inject more than 2 ml. of solution or emulsion per 20 Gm. mouse. In general, materials which caused death from injection of 10 mg. or less were considered unsuitable.

Hemolysis Test in Mice.—Mice which died within five minutes after intravenous injection of the solutions were decapitated and bled into 5% U. S. P. dextrose. This mixture was then centrifuged and the supernatant fluid was observed for the presence of hemoglobin. If the mouse did not die within five minutes, it was stunned by a blow on the head and then decapitated, etc.

Chronic Rat Toxicity Test.—This is a modification of the test described by Geyer, *et al.* (27). Albino rats weighing 180–244 Gm. were divided into groups of 4 rats each and injected via the tail vein for five consecutive days with the control or unknown material at a dose of 1 ml. per 100 Gm. of rat. Seven hours following the fifth injection, 2 rats from each group were killed with an excess of Nembutal® given intraperitoneally and portions of the lungs, liver, and kidneys were removed for study. Representative samples of these organs

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Acknowledgment is gratefully made to H. C. Spruth for counsel and aid in conducting certain animal tests.
[1] Atlas Powder Co., Wilmington, Del.
[2] Cherry-Burrell Co., Chicago, Ill.

were fixed in 10% formalin. Paraffin sections were prepared and stained with Mayer's acid hemalum and counterstained with eosin. Frozen sections of formalin-fixed materials were stained for fat with Sudan IV. Five days after the last injection this procedure was repeated on the remaining 2 rats from each group.

Blood Analyses in Dogs.—*A. Fat.*—The blood fat level was determined by the method of Geyer, Mann, and Stare (28) using an Evelyn Photoelectric Colorimeter with a 470 mμ filter.

B. Hemoglobin.—The usual "acid hematin" method with visual comparison with a glass color standard was used for determining blood hemoglobin.

Kidney Function Test in Dogs.—The phenolsulfonphthalein was given intravenously at a dose of 0.4 mg. per Kg. body weight.

Liver Function Test in Dogs.—The Bromsulphalein® was given intravenously at a dose of 5 mg. per Kg. body weight.

Dog Experiments.—Adult mongrel female dogs were used. Dogs 68 and 69 were injected intravenously with the fat emulsions at a dose of 15 ml. per Kg. body weight, given over a period of one hour. They consumed the customary kennel ration *ad libitum*.

With dogs 71, 72, and 73 the injections were increased to 30 ml. per Kg. body weight given over a period of ninety minutes. To eliminate the possibility of a loss of appetite affecting the results, the dogs were given a diet that furnished adequate amounts of all essentials and calories. Forced feeding was used when necessary to insure complete consumption of the day's ration. Since the emulsion furnished 48 calories per Kg. body weight per day the diet was so constructed to furnish an additional 35 calories per Kg. This is in the range of adequate caloric intake for the dog as recommended by Cowgill (29). The diet was fed at a dose of 10 Gm. per Kg. of body weight, and furnished daily per Kg. of dog the following: 1 Gm. dried beef blood fibrin, 4 Gm. sucrose, 3.7 Gm. white corn dextrin, 0.6 Gm. salt mixture (30), 0.25 Gm. agar, the amount of liver fraction 0[3] equivalent to 0.3 Gm. of 1 to 20 liver powder, 40 mg. wheat germ oil, 40 mg. choline chloride, 70 mg. of a concentrate containing 55,000 U. S. P. units of vitamin A and 5,500 U. S. P. units of vitamin D per gram, 0.74 mg. inositol, 0.015 mg. para-aminobenzoic acid, 0.09 mg. thiamin·HCl, 0.09 mg. riboflavin, 1.8 mg. nicotinamide, 0.06 mg. pyridoxine·HCl, 0.27 mg. *dl*-calcium pantothenate, 0.45 μg. biotin, 15 μg. folic acid, 1 μg. vitamin B_{12}. In addition 2 Iberin® Ferrous Capsules[4] were fed daily to each dog to provide 96 mg. of iron plus added amounts of B vitamins and liver concentrate.

RESULTS

Comparative Toxicity of Emulsifiers.—The results of the mice tests on various emulsifying agents are presented in Table I. As a basis for comparison, it should be mentioned that an injection of 2 ml. per 20 Gm. mouse of either 5% U. S. P. dextrose, isotonic saline, or freshly prepared emulsions containing 15% corn oil, 1% Glidden alcohol-soluble phosphatide,[5] and 5% U. S. P. dextrose was neither toxic nor hemolytic.

The data in Table I suggest that Tween® 60, 61, and 65 are much less toxic with regard to mouse toxicity and hemolysis than the other compounds tested.

A 0.5% solution of Tween® 60 in isotonic saline was also nonhemolytic to human blood *in vitro*.

Comparative Emulsifying Properties.—Many emulsifiers were rejected because emulsions made from them were either unstable initially or broke badly after autoclaving at 15 p. s. i. for fifteen minutes. We wish to emphasize that the emulsifying properties required for an intravenous product are of a highly specialized nature. Rejection of an emulsifier for this purpose does not reflect on its suitability for other uses. Preferably the concentration of emulsifying agent should be kept low in an intravenous preparation. Therefore, we tested all emulsifiers at concentrations of 3% or less, usually 2%.

The following emulsifiers were eliminated by such stability tests: (the numbers in parentheses are the per cent w/v of emulsifier used) Victawet® 12 (1),[6] Victamul® 116-B (1),[6] Good-rite® N-dodecyl-β-alanine (2),[7] Glyceryl monostearate 2210 (3),[8] Triton® A-20 (2)[9], Triton® X-100 (2),[9] Polyvinylpyrrolidone (1),[10] Tween® 81 (1),[11] Tween® 85 (2),[11] Tween® 40 (2),[11] Tween® 80 (2),[11] Kraystay® Type A (1),[12] Kraystay® Type Z (1),[12] and Kraystay® Type E (0.5).[12]

Gelatin.—Our experiences with Ucopco gelatin[13] as an emulsifier present a special case. As measured by the mouse toxicity test, emulsions containing corn oil and gelatin were nontoxic before autoclaving, but became toxic after autoclaving. Concentrations of gelatin were varied from 2 to 6%, and of corn oil from 10 to 15% without affecting the results. The size of the gelatin molecule is known to decrease during autoclaving. This change may in some way cause the autoclaved emulsions to be less stable *in vivo* than the original unautoclaved emulsions.

Dr. Paul R. Cannon, of the University of Chicago, reported to us that the aseptically prepared gelatin emulsions which we supplied him caused death in adult protein-depleted rats when given by intravenous drip at a dose of 6 ml. twice a day. After death, such animals had huge white livers and spleens and dilated and congested lungs. Pulmonary fat emboli were present in the lungs. Dr. Cannon suggests that the fat particles were removed in the liver and spleen by the phagocytic cells and that coalesced particles within the phagocytes broke out again into the blood stream and flooded the lungs

[3] Wilson Laboratories, Chicago, Ill.
[4] Abbott Laboratories, North Chicago, Ill.
[5] The Glidden Company, Chicago, Ill.
[6] Mixed neutral alkyl phosphates, Victor Chemical Co., Chicago, Ill.
[7] N-Dodecyl-β-alanine, B. F. Goodrich Chemical Co., Cleveland, Ohio.
[8] Emery Industries Inc., Cincinnati, Ohio.
[9] Alkylated aryl polyether alcohols, Rohm & Haas Co., Philadelphia, Pa.
[10] General Aniline, New York, N. Y.
[11] Tween 80, polyoxyethylene sorbitan monooleate, Tween 81, polyoxyethylene sorbitan monooleate, Tween 40, polyoxyethylene sorbitan monopalmitate, Tween 85, polyoxyethylene sorbitan trioleate, Atlas Powder Co., Wilmington, Del.
[12] Dehydrated gelose extract of Irish moss, Phenix Pabst-Ett Co., Chicago, Ill.
[13] Nonpyrogenic gelatin, United Chemical and Organic Products, Division of Wilson and Co., Chicago, Ill.

TABLE I.—TOXICITY TESTS ON EMULSIFYING AGENTS

Trade Name	Chemical Type	Form in which Tested	Intravenous Mouse Toxicity mg. per 20 Gm. mouse	Remarks
Aldo 28[a]	Glyceryl monostearate	Emulsion 1% with 15 % oil		Slight *in vitro* hemolysis
Triton A-20[b]	Alkylated aryl polyether alcohol	0.75% solution in 5% dextrose	0 of 5 died at 15 mg.	See effects on blood fat levels (31)
Demal 14[c]	Polyhydric alcohol fatty acid ester	1% solution in 5% dextrose	0 of 3 died at 5 mg. 1 of 3 died at 10 mg. 3 of 3 died at 20 mg.	All samples tested were pyrogenic (U. S. P method)
Tween 60[d]	Polyoxyethylene sorbitan monostearate	Emulsion 1% with 10% oil	0 of 4 died at 10 mg. 0 of 3 died at 15 mg. 2 of 4 died at 20 mg.	
Tween 60[d]	Polyoxyethylene sorbitan monostearate	2% solution in 5% dextrose	0 of 3 died at 40 mg.	No *in vivo* hemolysis
Tween 61[d]	Polyoxyethylene sorbitan monostearate	Emulsion 2% with 15% oil	0 of 3 died at 20 mg. 3 of 3 died at 40 mg.	
Tween 65[d]	Polyoxyethylene sorbitan tristearate	Emulsion 1% with 15% oil	0 of 3 died at 20 mg.	
Tween 80[d]	Polyoxyethylene sorbitan monooleate	2% solution in 5% dextrose	0 of 3 died at 20 mg. 0 of 3 died at 20 mg.	*In vivo* hemolysis at 40 mg. dose
Tween 81[d]	Polyoxyethylene sorbitan monooleate	2% solution in saline	3 of 3 died at 40 mg. 3 of 3 died at 40 mg.	Extreme *in vivo* hemolysis
Methocel[e]	Methylcellulose	2% solution in 5% dextrose	3 of 3 died at 20 mg.	
Kraystay Type D[f]	Calcium salt of sulfuric acid esters of a colloidal carbohydrate complex	Emulsion 1% with 15% oil	2 of 3 died at 5 mg. 3 of 3 died at 7.5 mg.	
Kraystay Type X[f]	Calcium salt of sulfuric acid esters of a colloidal carbohydrate complex	Emulsion 0.75% with 15% oil	3 of 3 died at 3.75 mg.	
...	Distearoyl citrate	Emulsion 2% with 15% oil	3 of 3 died at 10 mg.	
Emulphor ON[g]	Polyethylene ether of an oleyl alcohol	Emulsion 1% with 15% oil	1 of 3 died at 10 mg.	
Igepal CA[g]	Polyethylene ether of isooctyl phenol	1% solution in 5% dextrose	3 of 3 died at 10 mg.	
...	Oleyl peptides	Emulsion 0.37% with 15% oil	3 of 3 died at 3.7 mg.	
Marasperse N[h]	Sodium lignosulfate	1% solution in 5% dextrose	3 of 3 died at 10 mg.	
Marasperse CB[h]	Sodium lignosulfate	1% solution in 5% dextrose	3 of 3 died at 10 mg.	

[a] Glyco Products Co., Inc., Brooklyn, N. Y. [b] Rohm and Haas Co., Philadelphia, Pa. [c] Emulsol Corp., Chicago, Ill. [d] Atlas Powder Co., Wilmington, Del. [e] Dow Chemical Co., Midland, Mich. [f] Phoenix Pabst-Ett Co., Chicago, Ill. [g] General Dyestuff Corp., New York, N. Y. [h] Marathon Corp., Rothschild, Wis.

TABLE II.—HEMATOLOGICAL AND CHEMICAL DATA ARISING FROM DAILY INJECTIONS OF TWEEN 60 EMULSIONS[a]

Date	Leucocyte Count, ×10³			Erythrocyte Count, ×10⁶			Hemoglobin, Gm. %			Blood Nonprotein, mg. %			Plasma Protein, Gm. %			Liver Function, Retention after ½-Hour		% Dye	Kidney Function, % Dye Excretion within 1 Hour		
	Dog 71	Dog 72	Dog 73	Dog 71	Dog 72	Dog 73	Dog 71	Dog 72	Dog 73	Dog 71	Dog 72	Dog 73	Dog 71	Dog 72	Dog 73	Dog 71	Dog 72	Dog 73	Dog 71	Dog 72	Dog 73
10-21[b]	10.2	9.8	11.8	7.42	6.61	5.44	16.5	12.9	14.0	38	35	33	5.5	5.7	6.2	5	5	5	74	74	69
11-5[c]	19.6	14.7	16.6	7.35	7.70	6.55	15.5	12.5	15.0	42	32	37	6.1	5.8	6.1	5	5	<10	..	..	..
11-20[d]	..	..	14.6	..	..	7.75	..	..	16.0	..	..	29	..	..	5.8	..	..	5	..	..	..
12-10[e]	..	..	..	..	..	..	..	..	..	38	38	..	5.5	5.7	..	..	..	..	75	70	..
12-11	18.2	28.9	..	6.73	5.91	..	14.0	12.5	..	..	..	..	..	..	..	5	5	..	..	..	..

[a] Emulsion contained 15% coconut oil, 0.5% Tween 60, 5% U. S. P. dextrose, 0.01% mixed tocopherols, and 79.5% water. Emulsion was injected at a total dose and rate of 30 ml. per Kg. body weight over ninety minutes.
[b] Control values before injections.
[c] Dogs 71 and 72 were injected daily during the periods 10-22 through 10-26 and 10-29 through 11-2. Dog 73 was injected daily during the periods 10-22 through 10-26 and 10-29 through 10-31, and on 11-2.
[d] Dog 73 was injected daily during the period 11-6 through 11-9 and on 11-12.
[e] Dogs 71 and 72 were injected daily during the periods 11-6 through 11-8, 11-19 through 11-23, 11-26 through 11-30, 12-3 through 12-7, and on 12-10.
Electrocardiogram records were made during the injections for all dogs on 10-30. For dogs 71 and 72, records were also made on 12-10.

so quickly with large globules of fat that pulmonary embolism occurred.

Autoclaved gelatin emulsions caused death in dogs when given intravenously. This occurred even for certain gelatin emulsions not autoclaved, but prepared under sterile conditions.

Tween® 60.—Tween® 60, 61, and 65 (the polyoxyethylene sorbitan stearates) are all good emulsifiers and comparatively nontoxic in mice. Because Tween® 61 and 65 are not water-soluble, they must first be incorporated into emulsions before pharmacological tests are possible. Such a procedure makes it difficult to ascertain whether the effects observed arise from action of the emulsifier *per se*, or the emulsion. It is for this reason that we have concentrated our studies upon the water-soluble Tween® 60. Because the pharmacological effects of intravenously administered Tween® 60 were largely unknown, we confined our investigations to the minimum concentration necessary for stable emulsification, i. e., 0.5% w/v.

A. Stability to Storage.—The autoclaved Tween® 60 emulsions (10% fat) had an average particle size of 1 micron or less, with relatively few particles as large as 2 to 3 microns. The freshly prepared emulsions killed no mice at 1 ml. per 20 Gm. and when stored at 40° showed no increase in mouse toxicity even after seven months. The time interval after which they might have become toxic to mice is not known as the tests were discontinued after seven months' storage.

We have observed a slow hydrolysis of Tween® 60 when 0.5% aqueous solutions are stored at 40°. This was determined in a manner analogous to that used by Davis (32) for Tween® 80. However, no change in stability, no increase in mouse toxicity, nor effects on the blood pressure of the monkey developed after long storage of Tween® 60 emulsions.

B. Chronic Rat Toxicity Tests.—This test as described in Methods was applied to 3 groups of rats receiving respectively: 5% U. S. P. dextrose; 0.5% Tween® 60 in 5% U. S. P. dextrose; and an emulsion prepared under aseptic conditions containing 15% corn oil, 0.5% Tween® 60, and 5% U. S. P. dextrose. There were no significant changes in any of the organs examined that could be attributed to the Tween® 60 or the emulsions.[14]

C. Chronic Dog Injections.—We next determined the effects of successive daily intravenous injections of aseptically-prepared Tween® 60-stabilized emulsions upon dogs. The following were studied: blood components, liver and kidney function, internal organs as revealed by autopsy, and the rate of disappearance of injected fat from the blood stream.

1 Preliminary Experiments.—It was found that intravenously-administred fat rapidly disappears from the blood after the first injection and this rate of removal is not impaired after 17 successive daily injections (Fig. 1, Dog 69). At all times, the blood fat concentration was zero within twenty-four hours after the injection. Although no control values were determined, blood analyses were run after 21 successive daily injections. The following values were obtained: leucocytes 15,200, erythrocytes 7,720,000, hemoglobin 14.5 Gm. per cent,

[14] We are indebted to Dr. F. R. Goble for the preparation and examination of these tissues.

nonprotein nitrogen 31 mg. per cent, liver function dye retention 5% in one-half hour. These are all within the normal range.

Similar blood fat results were obtained on Dog 68, which received the emulsion for nine successive days.

II Long Term Experiments.—Three dogs (Dogs 71, 72, and 73) were intravenously injected daily, except for week ends, with an aseptically-prepared coconut oil emulsion. At various intervals the blood analyses and other tests were run. Due to the frequent puncturing of leg veins to obtain all of these samples, it became impossible after approximately three weeks to continue the daily emulsion injections until a supply of polyethylene medical tubing could be obtained to permit use of the juglar veins in a manner similar to that described by Kingma (33). This is the explanation for the ten-day period of no injections (Nov. 8–19), as indicated in Table II. No detrimental effects arising from the emulsion could be detected by the blood analyses or liver and kidney function tests as tabulated in Tables II and III. As shown in Fig. 1, the rate of removal of injected fat from the blood was no different after many successive daily injections than that observed on the first day of injections. On certain days, as indicated in Table II, electrocardiographic studies were performed prior to, during, and subsequent to the infusion of the emulsion.[18] No remarkable changes were noted in these records as a result of the immediate or continued infusion of the emulsions.

At the termination of the experiments the dogs were killed by an excess of Nembutal® given intravenously and portions of the heart, lung, liver, spleen, kidneys, adrenal and thyroid glands, pancreas, stomach, and intestine were removed for

TABLE III.—LEUCOCYTE DIFFERENTIAL COUNT ON DOGS RECEIVING DAILY INJECTIONS OF TWEEN 60 EMULSIONS

	Date	Number of Daily Injections Dog has Received	Nonseg. Neutro.	Seg. Neutro.	Eosin.	Baso.	Lymph.	Young Lymph.	Mono.	Young Nonseg. Neutro.
Dog 71	10-21	Control	2	74	1	0	23	0	0	0
	11- 5	10	15	66	2	0	16	1	0	0
	12-10	28	21	58	7	0	10	2	0	0
Dog 72	10-21	Control	4	74	1	0	21	0	0	0
	11- 5	10	11	64	2	0	20	3	0	0
	12-10	28	18	65	10	0	5	0	1	1
Dog 73	10-21	Control	2	84	1	0	13	0	0	0
	11- 5	9	17	73	0	0	10	0	0	0
	11-20	14	21	52	4	0	20	2	1	0
Dog 69		20	30	49	3	0	11	4	2	1

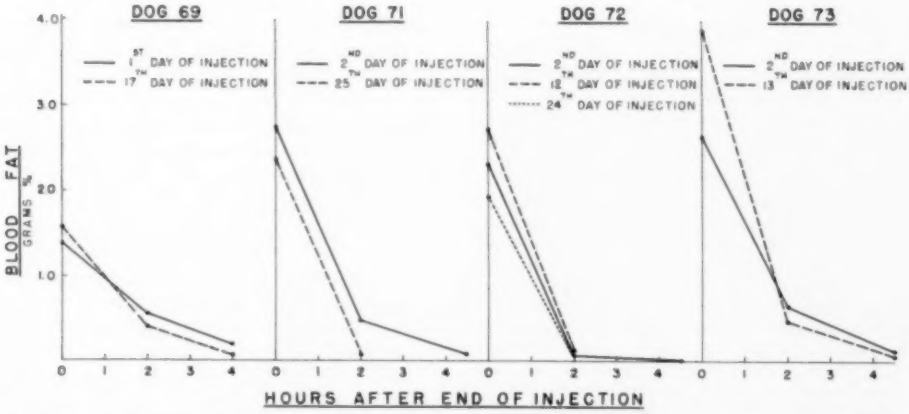

Fig. 1.—Dog blood fat levels following daily intravenous injections of Tween 60 emulsions. Dog 69 received an emulsion containing 15% corn oil, 0.5% Tween 60, 5% U. S. P. dextrose, and 79.5% water at a total dose and rate of 15 ml. per Kg. body weight per hour. Dogs 71, 72, and 73 received an emulsion containing 15% coconut oil, 0.5% Tween 60, 5% U. S. P. dextrose, and 79.5% water at a total dose and rate of 30 ml. per Kg. body weight per hour.

[18] We are indebted to Dr. J. L. Schmidt for performing the electrocardiographic studies.

study. These tissues were prepared and stained by the procedure described in Methods for rat tissues. No pathological deviation from normal was observed in any of the organs.[18]

D. Effect on Blood Pressure.—Krantz, *et al.* (34), have described a prolonged fall in blood pressure produced in dogs by intravenous injection of solutions of Tween® 20, 60 or 80. He has found this to be an allergic response limited to the dog and certain other members of the genus *Canis*. According to Krantz other laboratory animals, or man, appear to enjoy an immunity to this allergic-type response.

Schmidt, Goodsell, and Richards (35) have reported that continuous infusions of 0.5% Tween® 60 solutions in rabbit, monkey, and man caused no obvious change of circulatory status. They have, however, observed certain pharmacodynamic disturbances in the dog and cat following the injection of Tween® 60 solution. It is of interest to note that the Harvard group (36) has reported that cats are peculiarly sensitive to a vasodepressor substance present in their phosphatide emulsifier.

Work in the Pharmacology Department of these Laboratories (15) indicated no untoward circulatory effects in monkeys injected with fresh or old Tween® 60 fat emulsions. This result, together with the results in man with Tween® 60 alone (35) encouraged cautious trial of the emulsion in humans. Schmidt, *et al.* (15), report that three of four individuals receiving this emulsion experienced flushing of the face, epigastric or substernal distress, and cough after only a few ml. of the emulsion had been injected.

DISCUSSION

The detailed pharmacology of Tween® 60 and Tween® 60-stabilized emulsions in various species and the investigation in humans is not within the scope of this paper. We do not yet know the ultimate fate of Tween® 60 in the body. When we learned that Tween® 60 itself did not cause a circulatory response in man (35) analysis was made of the urine samples from these subjects. The few analytical data obtained were uncertain and erratic so that the balance data were not meaningful. In spite of these limitations, the data suggest that excretion is rapid, i.e., one-third within fifteen minutes after completion of the injection. Whether the material in the urine is the intact Tween® molecule or only the polyoxyethylene chain is not clear. These excretion studies are preliminary and the results obtained tentative.

In evaluating the Tween® series for intravenous toxicity, we found the stearic acid esters of polyoxyethylene sorbitan (Tween® 60, Tween® 61, and Tween® 65) considerably less toxic to mice than the rest of the series. The Tweens differ only in the number and type of fatty acids and in length of the polyoxyethylene chain. Thus the reason for this difference in toxicity is not clear.

The Ucopco gelatin used in our studies is a special nonpyrogenic gelatin prepared from pig skin. Our gelatin was, therefore, not comparable to that (Knox P-20 gelatin prepared from bone) used by Shafiroff, *et al.* (13). It is a matter of record that different gelatins may vary considerably in their chain length and resistance to degradation. Both Shafiroff's group and ourselves prepared gelatin emulsions by using sterile techniques. However, our preparation involved the use of considerably more heat than did Shafiroff's, which may explain the apparent difference in the toxicity of these emulsions.

[18] We are indebted to Dr. F. R. Goble for the preparation and examination of these tissues.

SUMMARY

1. Fat emulsions designed for intravenous therapy must fulfill many safety and stability requirements when designed for commercial distribution. Various emulsifying agents have been evaluated from this viewpoint.

2. Preliminary studies of the properties of Tween® 60 indicated that this emulsifier warranted detailed investigation. Oil-in-water emulsions containing 10 per cent vegetable oil and 0.5 per cent Tween® 60 are stable to autoclaving and can be stored at 40° for seven months or longer with no increase in toxicity to animals. Tween® 60-stabilized emulsions are nontoxic intravenously as measured by the acute mouse and chronic rat tests. The emulsions were given daily to dogs for several weeks with no effect on blood components or liver and kidney function. There were, however, certain acute pharmacodynamic effects caused by the injection of Tween® 60-stabilized emulsions which did not encourage widespread clinical trial of such emulsions. The unsuitability of these emulsions for use in the human was clearly established when it was found that patients reacted unfavorably to only small initial injections.

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The Comparative Anatomy of Certain Species of *Smilax**

By JAMES D. CAPONETTI and MAYNARD W. QUIMBY†

A comparative anatomical study of the vegetative organs of five native species of *Smilax* is reported upon. Particular attention is given to histological features of the roots, the leaves, the aerial stems, and the rhizomes, which aid in distinguishing one species from another.

THE PURPOSE of this research was to carry out a study of the comparative anatomy of the foliage leaves, the roots, the aerial stems, and the rhizomes of five species of *Smilax*. The species considered here are as follows: *Smilax auriculata* Walt., *S. hispida* Muhl., *S. glauca* Walt., *S. bona-nox* L., and *S. herbacea* L.

A decision to undertake this problem was made after it was determined that there is little information in the literature about the anatomy of species of *Smilax*. Only the species which yield the drug sarsaparilla have been studied anatomically and the results reported. Sarsaparilla is the only crude drug in general use today that is yielded by this genus.

A number of workers in the field of mycology have reported on various fungi found growing on species of *Smilax*. Other investigators have reported on some of the American species as forage plants of importance to certain wild animals. Coker (1) has reported on the various foods and articles of craftsmanship that have been prepared from species of *Smilax*.

Smilax, a genus of liliaceous plants, consists of about three hundred species for the entire world (2). Many plants of this genus are known as "greenbriers" or "catbriers." They are shrubby or herbaceous plants of the Tropics, of North America, of the Mediterranean region, and of eastern Asia, and are usually climbing or are supported by tendrils. According to Bailey (3) about twenty-five species are native to the United States. Eleven species and a number of varieties are included in Gray's Manual of Botany, eighth edition (4).

The authors wish to thank Dr. Carl H. Johnson of the University of Florida, Dr. Carl C. Albers of the University of Texas, and Dr. George M. Hocking of Alabama Polytechnic Institute, for supplying some of the plant material for this work. Other material was collected in the vicinity of Boston by the authors. All materials were preserved in 50% ethyl alcohol containing a small percentage of glycerin. Sections for microscopic study were prepared by the usual microtechnical methods. Temporary and permanent mounts were employed.

Preserved material and slides of all species worked upon have been deposited in the botanical collections of the Massachusetts College of Pharmacy.

ANATOMY

Roots

General Anatomy.—Transverse sections of the roots (Figs. 1 and 2) of all species studied show typical monocotyledonous root structure. The epidermis of each of the species consists of a layer of

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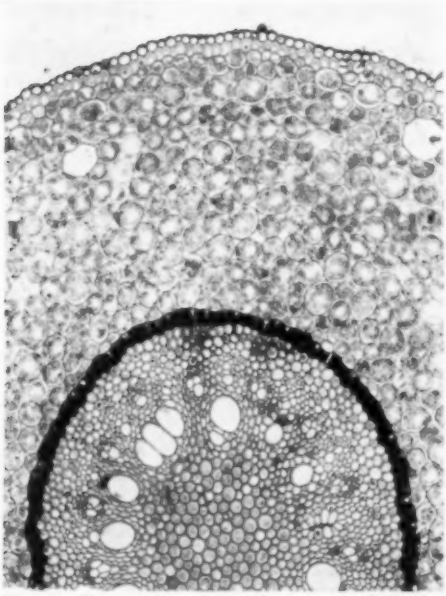

Fig. 1.—Transverse section of the root of *Smilax herbacea* L.

cells typical of such tissue. In general the cortex is a rather broad zone of parenchyma cells usually containing starch grains. Cells of the endodermis are distinctive (Fig. 3). Their inner and radial walls are thickened, and pore canals are rather distinct. In each species the pericyclic region consists of cells with thickened walls, and the vascular tissue exhibits the polyarch radial arrangement commonly seen in roots of the monocotyledons. The central core of pith consists of relatively thick-walled parenchyma cells containing starch grains.

Comparative Anatomy.—Of the five species studied only the root of *S. herbacea* shows a distinct hypodermis. In this species this subepidermal zone consists of a single layer of rather characteristic cells. The cells vary somewhat in size and are commonly somewhat radially elongated. The outer walls of all these cells are thicker than the radial and inner walls. The lumina of these cells differ in sizes and occur toward the inner margins of the cells.

Scattered about in the cortical regions of *S. auriculata* and *S. hispida* are large and small secretory intercellular cavities. *S. glauca*, *S. bona-nox*, and *S. herbacea* do not possess these cavities. The inner walls of the innermost layer of cortical cells

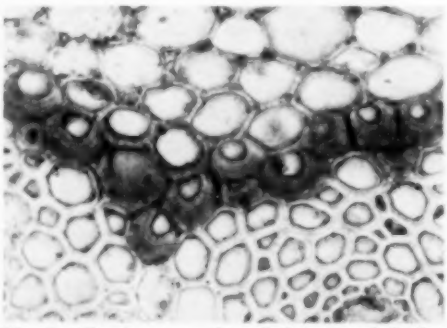

Fig. 3.—Transverse section of the root of *Smilax herbacea* L. showing endodermal cells.

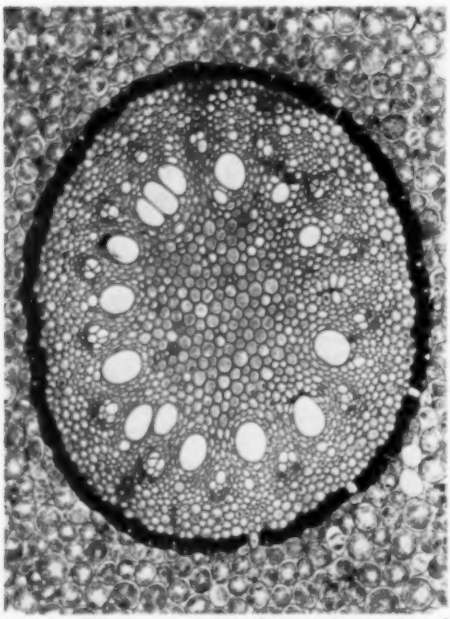

Fig. 2.—Transverse section of the stelar portion of the root of *Smilax herbacea* L.

of *S. auriculata*, *S. hispida*, *S. glauca*, and *S. bona-nox* are distinctly thickened and heavily lignified. This condition does not exist in *S. herbacea*.

Among the cortical cells of *S. herbacea* there are numerous, scattered cells containing columnar crystals (Fig. 1) but not containing starch grains. These cells are larger than the ordinary cortical cells containing starch grains. Cortical cells of the other four species do not contain these columnar crystals. Within the inner cortex of *S. hispida* there are numerous scattered, lignified, thick-walled cells. The inner cortices of the other four species do not contain such cells.

The endodermis is the most distinctive tissue in all the five species. The endodermal cells of *S. auriculata* are somewhat radially elongated and possess characteristically thick walls. The lumen is more or less triangular, and the pore canals are very distinct. The endodermal cells of *S. hispida* are more elongated radially than they are in *S. auriculata*, and they taper toward their inner ends. The lumina are small and more or less circular. Pore canals are very much less distinct than they are in *S. auriculata*. Some sections show endodermal cells with large lumina which are of almost the

same shape as the cells. These sections were cut from young roots. In *S. glauca* the endodermal cells are radially elongated, but not nearly so much as in *S. auriculata* and *S. hispida*. The lumina of the cells vary in shape, but in most cells are small and circular. In a few cells the lumen is elongated, but toward the outer margin of the cell. Such elongated lumina were not found in the endodermal cells of the other four species. Pore canals are indistinct in some cells and distinct in others. The endodermal cells of *S. bona-nox* are radially elongated but taper at the inner ends. Such tapering was not observed in *S. auriculata* and *S. glauca*. The lumina are small and irregularly circular in most of the cells. In a few cells the lumina are triangular. Pore canals are very distinct and can be clearly seen only in the inner wall. This condition was not observed in the endodermal cells of the other four species. In *S. herbacea* the endodermal cells are not radially elongated, as in the other four species. The cells are more nearly isodiametric and are slightly narrower at the inner wall. The lumina are small, triangular, and are situated slightly toward the outer margins of the cells. Pore canals are just visible. Occasionally the continuity of the endodermis is interrupted by a uniformly thin-walled cell (transfusion cell) (Fig. 2.). This condition was not observed in the other four species.

The radial dimension of the pericycle varies in the five species. *S. glauca* has the broadest pericycle. The second broadest is that of *S. hispida;* the third, of *S. bona-nox;* the fourth, of *S. auriculata;* and the fifth, of *S. herbacea*. The pericyclic cells of *S. glauca* are not so thick-walled as those of *S. auriculata* or of *S. hispida*. The pericyclic cells of *S. bona-nox* have extremely thick walls, thicker than those in any of the other four species.

The extent of the pith varies in the five species. *S. bona-nox* has the broadest pith; *S. herbacea*, the second broadest; *S. auriculata*, the third; *S. hispida*, the fourth; *S. glauca* the narrowest. The walls of the parenchyma cells of the pith are much thicker in *S. herbacea* than in any of the other four species.

Foliage Leaves

General Anatomy.—Reference to Figs. 4 and 5 will show that the upper and lower epidermises consist of single layers of more or less elongated cells. In a few instances squarish cells are present. Stomates

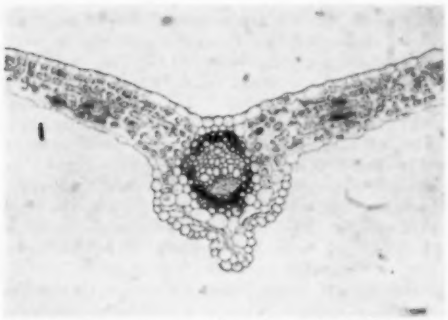

Fig. 4.—Transverse section of the leaf of *Smilax herbacea* L.

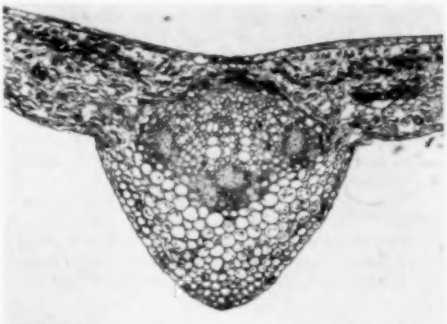

Fig. 5.—Transverse section of the leaf of *Smilax bona-nox* L.

are present but not easily discernible. The mesophyll is composed of thin-walled, nearly isodiametric cells compactly arranged. Here and there lateral veins can be seen in the mesophyll.

In transverse section the midrib appears more or less rounded. The vascular tissue, consisting of a patch of xylem above and a patch of phloem below, is enclosed by a more or less continuous band of sclerenchyma. The rest of the midrib zone consists of thin-walled parenchyma cells, except for a small patch of collenchyma above and below the vascular tissue.

Comparative Anatomy.—The number of cells making up the breadth of the mesophyll varies in the five species. Table I shows this variation:

TABLE I.—VARIATION IN BREADTH OF MESOPHYLL

Species	Number of Cells
S. auriculata	8–9
S. hispida	4–6
S. glauca	6–7
S. bona-nox	8–11
S. herbacea	5–6

The cells of the lower epidermis of *S. glauca* possess short, stout, blunt papillae. Such structures were not observed in the other four species.

There are slight variations in the outlines of the midribs of the five species. The outlines of the midribs of *S. auriculata* and of *S. bona-nox* are more or less rounded; those of *S. hispida* and *S. glauca* are more pointed; and the midrib of *S. herbacea* has a characteristic peculiar protrusion below. The lower epidermis of the midrib of *S. glauca* lacks the papillae which are present on the lower epidermis of the lamina. In the lower portion of the midrib of *S glauca*, near the epidermis, there are a few thick-walled, circular, lignified cells. Such cells were not observed in the same region of the other four species. In the lower portion of the midrib of *S. herbacea* the cells are misshapen and appear to be crushed. This condition was not observed in the other four species.

There are only slight differences in the orientation of the vascular tissue within the midribs of the five species. In *S. auriculata* the band of sclerenchyma

that surrounds the xylem and phloem extends all the way to the upper epidermis. This condition did not appear in the other four species. Surrounding the sclerenchyma band of the vascular tissue in *S. bona-nox* there are cells whose inner walls are thick and lignified, and whose outer walls are thin and nonlignified. This tissue appears very much like endodermis. None of the other four species showed this tissue.

Aerial Stems

General Anatomy.—Transverse sections of the aerial stems show typical monocotyledonous structure (Figs. 6 and 7). The epidermis of these is composed of a single layer of thick-walled, circular, to square, to rectangular cells with well-developed cuticles and numerous sunken stomates. In each species the hypodermis is distinct and generally consists of a single layer of circular thick-walled cells. In a few places a double layer can be seen. The cortex is a rather narrow zone of parencyhma cells. In some species the whole cortex is lignified; in others, only the inner cortex. Upper portions of the stems do not show an endodermis. However, basal portions near the rhizomes usually show a distinct and well-developed endodermis which generally consists of a single wavy layer becoming occasionally a double or triple layer of more or less isodiametric cells. The inner and radial walls of these cells are very much thicker than the outer walls. The lumina are often circular to triangular and are situated toward the outer margins of the cells. Pore canals are more or less distinct. The outer part of the stele consists of a continuous ring of sclerenchyma tissue. The individual cells are more or less isodiametric, thick-walled, and heavily lignified. Imbedded in the sclerenchyma ring there are numerous, small, closed collateral bundles. The rest of the stele is composed of numerous large, closed collateral bundles, each of which is surrounded by a sheath of sclerenchyma and is imbedded in a matrix of ground parenchyma. Toward the inside, the bundles become larger and the sclerenchyma sheath which surrounds them becomes less broad. The ground parenchyma cells are relatively thick-walled and heavily lignified.

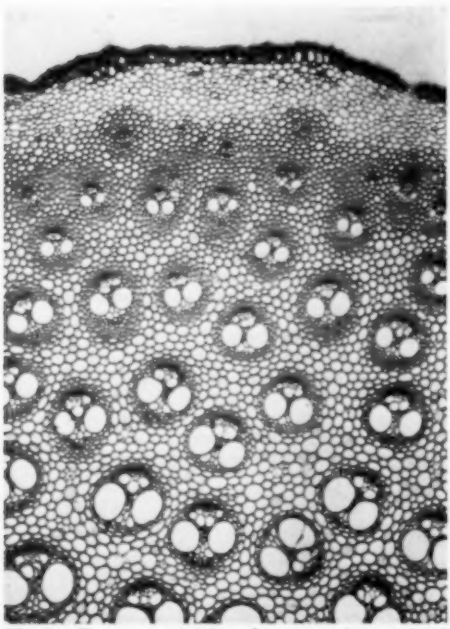

Fig. 6.—Transverse section of an upper internode of the aerial stem of *Smilax auriculata* Walt.

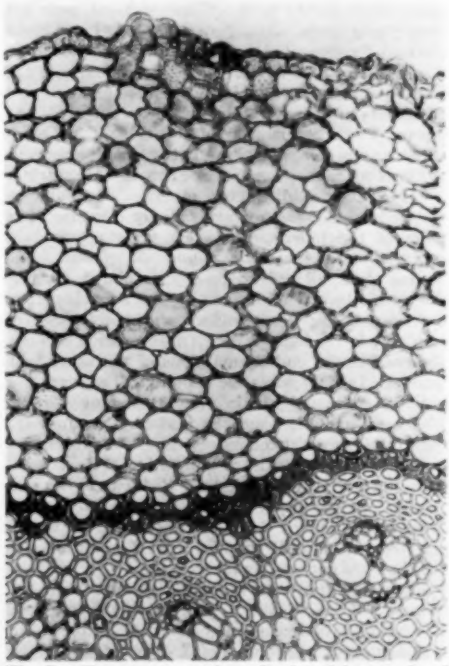

Fig. 7.—Transverse section of a basal internode of the aerial stem of *Smilax auriculata* Walt.

Comparative Anatomy.—The epidermal cells of *S. auriculata* and *S. bona-nox* are squarish, whereas those of *S. hispida* are rectangular, and those of *S. glauca* are hemispherical, i. e., the outer walls are straight and the inner and radial walls are rounded. The epidermal cells of the species named above are all thick-walled. The epidermal cells of *S. herbacea* are more or less isodiametric and thin-walled, and the cuticle is not so well developed as in the other four species.

The hypodermal cells of *S. auriculata*, *S. hispida*, *S. glauca*, and *S. bona-nox* are isodiametric and thick-walled. The lumina are of the same shapes as the cells. Those of *S. glauca* and *S. bona-nox* are lignified, whereas those of *S. auriculata* and *S. hispida* are not. A hypodermis was not observed in *S. herbacea*. Hypodermis was not distinct in basal portions of the stem in *S. auriculata* and *S. hispida*. However, in *S. glauca* and *S. bona-nox* the hypoder-

mis looks much the same as in upper portions of the stem.

The cortex of *S. auriculata* possesses numerous scattered, perfectly round, thick-walled cells which are larger than the regular cortical cells. The lumina vary in size, being sometimes extremely small. The cell walls vary in degree of lignification. The cortices of the other four species do not possess cells with these characteristics. All the cortical cells of *S. glauca* and *S. bona-nox* are heavily lignified. But in *S. auriculata*, *S. hispida*, and *S. herbacea* only the inner cortical cells, in from one to several layers, are lignified.

Basal portions of the stems of *S. auriculata*, *S. hispida*, *S. glauca*, and *S. herbacea* show an endodermis which is very similar in all four species. But in *S. hispida* the endodermis is occasionally as much as three cells thick. Basal portions of the stem of *S. bona-nox* did not show any endodermis.

The closed collateral bundles within the continuous ring of sclerenchyma in *S. herbacea* are almost of the same size as those in the rest of the stele. Within the continuous ring of sclerenchyma of the other four species, the closed collateral bundles are very much smaller than those in the rest of the stele.

The ground parenchyma cells of *S. auriculata*, *S. hispida*, *S. glauca*, and *S. bona-nox* are relatively thick-walled and possess heavily lignified walls. Those of *S. herbacea* are thin-walled, not lignified, and larger than those of the other four species.

Rhizomes

General Anatomy.—The rhizomes show at certain stages of growth some periderm formation. The epidermis consists of more or less isodiametric, thick-walled cells. A hypodermis was not discernible in any species. The cortex is a narrow zone of parenchyma cells containing starch grains. Within the cortex there are numerous scattered, yellowish mucilage cells which contain raphides of calcium oxalate. These cells are larger than the cortical cells and more nearly isodiametric. At the inner boundary of the cortex there is in some species an interrupted layer of endodermis; in others, a continuous zone of stone cells. The stele consists of many small, scattered, closed collateral bundles imbedded in a matrix of ground parenchyma. Each bundle is completely surrounded by a sheath of sclerenchyma. Within the ground parenchyma there are numerous scattered, yellowish mucilage cells which contain raphides of calcium oxalate and numerous starch grains.

Comparative Anatomy.—The epidermal cells of *S. bona-nox* are smaller than those of the other four species.

The cortical cells of *S. hispida*, *S. bona-nox*, and *S. herbacea* are lignified, whereas those of *S. auriculata* and *S. glauca* are not. Yellowish mucilage cells with raphides of calcium oxalate did not appear as abundantly in *S. hispida* as in the other four species. The yellowish mucilage cells of *S. glauca* are elongated, whereas in the other four species they are more nearly rounded.

The rhizomes of *S. auriculata* show neither an endodermal nor a stone cell zone. *S. hispida* lacks an endodermis, but does have a continuous stone cell zone which is two to six cells wide and which separates the cortex from the stele. The individual cells vary in size and shape. The walls are evenly thickened and show distinct pore canals. The lumina are large and of the same shape as the cells. *S. glauca* lacks a stone cell zone, but does possess an endodermis which consists of a single layer and in some places a double layer whose continuity is interrupted in some places. The individual cells are tangentially elongated. The inner and radial walls are very much thicker than the outer walls and the pore canals are very distinct. The lumina are tangentially elongated and are situated toward the outer margins of the cells. The cells contain a brownish substance. *S. bona-nox* lacks an endodermis, but does possess a stone cell zone similar to that of *S. hispida*. *S. herbacea* possesses both an endodermis and a stone cell zone. In that part of the rhizome in which there is an endodermis there is no stone cell zone and conversely where there is no endodermis there is a stone cell zone. The endodermis is usually of a single layer, but in some places a double layer occurs. In some places the continuity of the endodermis is interrupted by thin-walled cells. The individual endodermal cells are more or less isodiametric; the inner and radial walls are thicker than the outer wall; and the pore canals are distinct. The lumina are large, circular to triangular, and occur toward the outer sides of the cells. The stone cell zone is similar to those found in *S. hispida* and in *S. bona-nox*. Table II shows comparisons.

TABLE II.—COMPARISONS[a]

Species	Mucilage Cells	Raphides	Endodermis	Stone Cell Zone
S. auriculata	+	+	−	−
S. hispida	+	+	−	+
S. glauca	+	+	+	−
S. bona-nox	+	+	−	+
S. herbacea	+	+	+	+

[a] + Present, − absent.

The stele of *S. herbacea* possesses amphivasal bundles besides the regular closed collateral bundles. The steles of the other four species do not show amphivasal bundles.

Starch

Except for size, the character of the starch grains is similar in all five species. Most of them are simple, although a few are up to three-compound. The shape varies from perfectly circular to polygonal to truncate to reniform. The hilum is usually small and circular, but in some grains is elongated with branching clefts. The lamellae are distinct and concentric. Table III shows the comparative sizes of the starch grains.

TABLE III.—COMPARATIVE SIZES OF THE STARCH GRAINS

Species	Size	Average
S. auriculata	9–40 μ	25 μ
S. hispida	3–20 μ	15 μ
S. glauca	9–40 μ	25 μ
S. bona-nox	3–30 μ	20 μ
S. herbacea	3–10 μ	6 μ

SUMMARY

A report is given of the comparative anatomy of the foliage leaves, the roots, the aerial stems, and the rhizomes of five species of *Smilax*. The five species differ among themselves in the following respects: (*a*) all four organs of all five species show typical monocotyledonous structure, (*b*) the endodermal cells of the roots of each species are distinctive, (*c*) there are differences between the cortices of the roots, (*d*) there are differences in the radial dimensions of the pericycle in the roots, (*e*) the extent of pith varies in the roots, (*f*) the number of cells making up the breadth of the mesophyll varies in the leaves, (*g*) there are slight variations in the outlines of the midribs in the leaves, (*h*) there are slight differences in the orientation of the vascular tissue within the midribs of the leaves, (*i*) there are differences between the epidermal cells of the aerial stems, (*j*) the same is true for the hypodermises of the aerial stems, (*k*) basal internodes of the aerial stems show an endodermis; upper internodes do not show an endodermis, (*l*) some periderm formation was observed in the rhizomes, (*m*) within the cortex and the ground parenchyma in the rhizomes there are numerous scattered, yellowish mucilage cells, which contain raphides of calcium oxalate, (*n*) at the inner boundary of the cortex in the rhizomes there is in some species an interrupted layer of endodermis; in others, a continuous zone of stone cells, (*o*) except for size, the character of the starch grains in the rhizomes is similar.

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A General Study of the Effects of Lipids upon the Quantity of Peroxides Produced by Photosensitized Zinc Oxide*

By FREDERICK CLAIRE BLUBAUGH† and EARL P. GUTH‡

ZINC OXIDE is a common ingredient (1) of many medicinal products used in the treatment of a wide variety of dermatological conditions. Vehicles for such preparations consist mainly of the fixed oils, derived from plants or animals, or petrolatum.

Peroxides are produced by photosensitized zinc oxide in the presence of oxygen, water, and miscellaneous compounds (2). This study has been concerned with the effect of fixed oils upon the quantity of peroxides produced in liniments containing zinc oxide when exposed to ultraviolet light. Since calamine, U. S. P., is essentially zinc oxide, calamine liniment, N. F. IX, with modifications was selected as the preparation for study. In the study, calamine was replaced with zinc oxide, U. S. P., while other medicinal oils were substituted for olive oil.

The fixed oils selected for study were olive, expressed almond, peach kernel, lard, castor, cottonseed, sesame, linseed, and cod liver oil. Liquid petrolatum was also included.

The irradiation procedures employed by Reese and Guth (3), Mathias and Guth (4), and Minardi and Guth (5) on calamine lotion were modified to make them adapted to this study of the water-in-oil type of emulsions.

Chari and Quershi (6) found that sunlight, artificial ultraviolet and visible light up to 4,700 Å are effective in causing the reaction to proceed. They also reported increased yields

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of hydrogen peroxide in the presence of organic compounds. Yamahuzi, Nisioeda, and Ryusi (7), having studied the photochemical formation of hydrogen peroxide in the presence of biological sensitizers, suggest that the formation proceeds by three reactions:

$$2H_2O \rightarrow H_2O_2 + 2H \quad (I)$$

$$2H + O_2 \rightarrow H_2O_2 \quad (II)$$

$$A + H_2O + O_2 \rightarrow AO + H_2O_2 \quad (III)$$

"*A*" is the organic stabilizer. Although light energy is sufficient to promote these reactions, the heat produced by (II) and (III) would accelerate reaction (I) (8).

The presence of a small concentration of one of the many water-soluble easily oxidized compounds (termed "stabilizers," "promoters," or "additives") such as sodium formate, potassium oxalate, phenol, etc., in the zinc oxide–water-oxygen mixture increases the rate of peroxide formation greatly (9).

The presence of oxygen in the system is necessary for hydrogen peroxide formation. Hydrogen peroxide decomposition is very rapid in zinc oxide–water-additive mixtures in which the oxygen supply is low. These so-called "stabilizers" are actually reactants and undergo oxidation simultaneously with the hydrogen peroxide formation. The formate ion and the oxalate ion are oxidized to carbonate, while phenol is oxidized to catechol (9).

$$H_2O + O_2 + HCOO^- \rightarrow H_2O_2 + HCO_3^-$$

MATERIALS

The materials utilized in this study were as follows: Zinc oxide, U. S. P. (Merck & Co., Inc.), olive oil I U. S. P. (Gerber's pure virgin olive oil), olive oil II (a blend of the finest olive oils), expressed almond oil U. S. P., persic oil (peach), castor oil U. S. P., lard oil, cottonseed oil U. S. P., sesame oil U. S. P., linseed oil (raw), cod liver oil U. S. P., liquid petrolatum U. S. P.

EXPERIMENTAL

Preparation of Samples.—Calamine liniment, N. F. IX (10), was selected as the basic formula for the study of the effect of selected lipids upon the quantity of peroxides produced by photosensitized zinc oxide.

CALAMINE LINIMENT

Calamine	80 Gm.
Zinc oxide	80 Gm.
Olive oil	500 ml.
Calcium hydroxide solution, a sufficient quantity to make	1,000 ml.

Zinc oxide was substituted for the calamine so that the above formula was changed to zinc oxide 160 Gm., olive oil 500 ml., and calcium hydroxide solution, *q. s.*, to make 1,000 ml. In this phase of the study the only variable was the oil used.

The fixed oils used in dermatological preparations are from many species of plants and animals. They include the nondrying, semidrying, and drying oils of vegetable origin as well as those obtained from animals. The fixed oils selected for this study included representatives of each of the classes (11) of oils utilized in dermatological preparations. Each of the oils used was tested for purity and identity (Table I).

Each oil in an amount equal to olive oil in the formula for calamine liniment was mixed with zinc oxide. The zinc oxide–oil mixture was placed in an 8-fl. oz. prescription square and shaken for twenty minutes on a mechanical shaker. After the initial mixing, calcium hydroxide solution was added to the containers. The containers were closed immediately. This procedure maintained the concentration of the calcium hydroxide solution. The containers were returned to the mechanical shaker for an additional twenty minutes of shaking.

When the mixture consisted of two ingredients, i. e., zinc oxide and oil, zinc oxide and calcium hydroxide solution, or oil and calcium hydroxide solution, the same procedure of mixing and shaking was employed.

Five-gram samples of the zinc oxide liniments were used for irradiation. When only one or two of the components were studied, only that amount

TABLE I.—CHARACTERISTICS OF THE OILS AS DETERMINED BY ASSAY[a]

Oil	Class	Iodine Number	Saponification Number	Acid Number
Olive oil I[b]	I	84.9	193.4	0.97
Olive oil II	I	94.7	191.0	0.60
Expressed almond oil[b]	I	101.6	190.4	0.09
Peach kernel oil	I	103.6	189.9	1.09
Castor oil[b]	III	85.9	180.2	1.95
Lard oil	IV	36.5	39.8	6.29
Cottonseed oil[b]	V	110.3	194.0	0.04
Sesame oil[b]	V	111.1	188.4	0.22
Linseed oil	VI	154.5	191.8	4.80
Cod liver oil[b]	VII	150.7	186.1	1.19

[a] U. S. P. XIV methods of assay.
[b] Meet U. S. P. XIV specifications.

of the ingredient represented in a 5-Gm. sample of the zinc oxide liniment was irradiated. These amounts were determined on a weight basis. A 5-Gm. sample was made up of 0.76 Gm. of zinc oxide, 2.12 Gm. of oil, and 2.12 ml. of calcium hydroxide solution.

Samples for irradiation consisted of the following ingredients: Each ingredient separately, emulsions of the oils by addition of calcium hydroxide solution, zinc oxide in combination with the oils, zinc oxide in combination with calcium hydroxide solution, zinc oxide liniments.

Olive oil of two different grades is included in this study and these are designated olive oil I and olive oil II (see "Materials").

Method of Irradiation.—Five-gram samples of the zinc oxide liniments were weighed into 800-ml. Pyrex beakers. One hundred milliliters of distilled water or 100 ml. of 0.2 *M* sodium formate solution was added to the beakers. When only one or two of the ingredients were to be irradiated, the samples consisted only of the amount of those ingredients in a 5-Gm. sample.

The addition of sodium formate acted as an "additive" for the photochemical production of peroxides by zinc oxide. Its addition not only increased the production of peroxides (Table II) but acted as a "stabilizer," preventing marked destruction of peroxides. Hydrogen peroxide was destroyed at an appreciable rate in the absence of sodium formate.

The four 800-ml. beakers were placed in the water bath under the ultraviolet light. The water bath was cooled by running water. Glass stirrers, driven by a Cenco stirrer variable speed motor at 530 r. p. m., were utilized as a means of agitation of the samples during irradiation. The equipment was arranged so that each of the beakers was the same distance from the ultraviolet lamp. The distance from the lamp to the surface of the liquid was 35 cm.

The irradiation unit was completely enclosed within aluminum sheeting. The beakers rested on a false aluminum bottom in the water bath and were held in place by a sheet of aluminum containing holes that were the same diameter as the beakers. The level of the water in the bath was kept constant, slightly above the contents of the beakers.

Irradiation time for all samples was thirty minutes.

Method of Assay.—The iodometric method of Kolthoff (12) as modified by Chari and Quershi (2) was utilized for the determination of peroxides. Peroxides react with iodide in acid medium according to the following reaction: $H_2O_2 + 2I^- + 2H^+ \rightarrow I_2 + 2H_2O$. Ammonium molybdate catalyzes this reaction.

The irradiated samples were filtered immediately through Whatman No. 1 filter paper, giving a clear filtrate. The first 15 ml. of the filtrate was discarded. To a 50-ml. aliquot of the clear filtrate in an iodine flask, the following reagents were added in the order given: 10 ml. 2 *M* sulfuric acid, 10 ml. 0.2 *N* potassium iodide, 5 ml. 0.04 *N* ammonium molybdate.

The flasks were well shaken and placed in the dark for ten minutes. The liberated iodine was titrated with 0.04 *N* sodium thiosulfate, using freshly prepared starch solution as the indicator.

TABLE II.—COMPARATIVE TABULATION OF THE MICROGRAMS OF PEROXIDES PRODUCED BY ULTRAVIOLET IRRADIATION

		Irradiation in Distilled Water				Irradiation in Sodium Formate			
Oil	Class	Oils Alone, μg.	Oils + $Ca(OH)_2$, μg.	Oils + ZnO,[a] $Ca(OH)_2$, μg.	Oils + ZnO + $Ca(OH)_2$, μg.	Oils Alone, μg.	Oils + $Ca(OH)_2$, μg.	Oils + ZnO, μg.	Oils + ZnO + $Ca(OH)_2$, μg.
Olive oil I	I	73	81	141	131	159	100	276	1,634
Olive oil II	I	93	104	143	275	189	133	556	2,194
Express almond oil	I	117	128	201	384	162	167	3,871	3,210
Peach kernel oil	I	173	89	196	188	166	150	345	1,533
Castor oil	III	71	0	166	130	141	130	228	503
Lard oil	IV	139	164	149	122	236	220	206	196
Cottonseed oil	V	90	96	108	90	105	158	547	866
Sesame oil	V	112	121	173	555	185	183	632	3,036
Linseed oil	VI	181	188	138	175	201	182	368	218
Cod Liver oil	VII	237	112	254	181	289	304	357	1,182
Mineral oil		67	60	894	691	112	72	6,899	3,286

[a] ZnO = Zinc oxide (Merck).

DISCUSSION

The results of this investigation indicate that the quantity of peroxides produced by the irradiation of a system containing zinc oxide, lime water, and a lipid or liquid petrolatum with ultraviolet light will vary over a wide range. If some of the therapeutic value of such a system depends on this phenomenon, then the choice of lipid becomes very important. The observed variances have not been shown to be related to known properties of the lipids such as acid value or iodine number. The results show no correlation between the amount of peroxide formed and the acid value or iodine number. It is also impossible to predict just what quantity of peroxide any of the lipids will produce when in contact with other agents (Table II). It is interesting to note that expressed almond oil and sesame oil produce large quantities of peroxides and that these oils have been used in medicine since ancient times. A blend of olive oils gave higher results than pure

virgin olive oil U. S. P. There are some differences in the chemical characteristics of these two oils. The pure virgin olive oil gave an iodine number of 84.9, a saponification number of 193.4, and an acid value of 0.97. The blend of olive oil gave values of 94.7, 191.0, and 0.60 (Table I). It is possible that the higher unsaturation of the blend of olive oils was responsible for the difference in peroxide production. Peach kernel oil and expressed almond oil show approximately the same chemical values yet there was twice as much peroxide produced by the almond oil than was produced by the peach kernel oil. This fact would suggest that the two oils are not interchangeable in pharmaceutical preparations. The exceptionally high peroxide value obtained when liquid petrolatum was used in the liniment is noteworthy. Linseed oil, cottonseed oil, and lard oil all show relatively low values in peroxide formation.

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Metal Chelates of Alloxan*

By WINTHROP E. LANGE† and WILLIAM O. FOYE‡

Alloxan has been found to undergo chelation with Co^{++}, Ni^{++}, and Cu^{++} ions in aqueous solution, giving products having a 1:1 ratio of metal to alloxan. The isolated chelates were found to elicit an increase in blood sugar in rabbits comparable to that from alloxan itself, but over a much longer period of time. Metal chelates of two derivatives of alloxan, riboflavin, and its dichloro analog, gave greater increases in blood sugar, which suggests that alloxan may exert its diabetogenic effect as a metal chelate.

THE MECHANISM of action by which injections of alloxan produce a metabolic state resembling diabetes mellitus in animals (1) is still unknown, although a number of explanations for its action have been suggested. Gross observations of the pancreas after alloxan injection revealed that the beta cells were necrotized (2), possibly through overstimulation to produce insulin, but the role of alloxan was not clarified. It was later suggested by Cori and Colowick (3), after *in vitro* studies, that hexokinase activity was inhibited by diabetogenic substances, including alloxan, and restored by insulin, but others (4), also on the basis of *in vitro* observations, concluded that hexokinase activity was not impaired in alloxan-induced cases of diabetes. Stadie, *et al.* (5), reported, however, that the ability of the diaphragm to combine with insulin was diminished after administration of alloxan to rats. Alloxan inactivation of coenzyme A has been recently reported (6), with the suggestion that the diabetogenic effect of alloxan may in part be due to its reactivity with the sulfhydryl group of coenzyme A in the beta cells of the pancreas. In any case, it appears that alloxan combines with an enzyme or other essential substance which is ordinarily free to combine with substances such as insulin.

In connection with other work, we had occasion to prepare several metal chelates of alloxan, and these were subjected to routine blood-sugar determinations in rabbits. As shown in Table I, the administration of nondiabetogenic doses of these chelates produced increases in the blood sugar level comparable to that from alloxan itself, but the period of action was much longer. Greater increases resulted from administrations of metal chelates of some alloxan derivatives, namely riboflavin and the dichloro analog of riboflavin. In the latter cases, the differences

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The authors are extremely grateful to Drs. K. K. Chen and M. A. Root of the Lilly Research Laboratories for carrying out the blood sugar tests on the cobalt chelates of alloxan and the dichloro isoalloxazine, and to Dr. G. C. Gross of South Dakota State College for assisting with the other blood sugar tests.

between the blood sugar increases caused by the chelates and the nonmetallized agents is striking; riboflavin has no appreciable effect by itself (8), and the related isoalloxazine has a substantially lower effect than alloxan. These observations suggest that the chelates may be the active agents in producing blood sugar increases, although the possibility exists that alloxan could be stabilized through chelation and then slowly released to exert its characteristic effect. Seligson (7), for instance, demonstrated that alloxan is 40% converted to the inactive alloxanic acid in neutral, unbuffered, aqueous solution at 25° within a period of five minutes.

Combination of alloxan with sulfhydryl groups has been clearly demonstrated by Lazarow, who found that combinations of alloxan with cysteine or glutathione (9) or with 2,3-dimercaptopropanol (10) were nondiabetogenic. If alloxan exerts its diabetogenic effect through combination with the sulfhydryl group of an essential protein or enzyme such as hexokinase or coenzyme A, as previous work has indicated, then heavy metal chelates of alloxan should provide a more stable linkage with sulfhydryl groups and produce a longer action, since the metal chelated with alloxan is still available for complex formation with the sulfhydryl group. It is interesting to note, in this connection, that the cobalt salt of alloxanic acid, which should also exist as a chelate structure, exerted no blood sugar raising at all. This result is not surprising, in view of the fact that the pyrimidine structure, considered essential for diabetogenic activity (11) is no longer present.

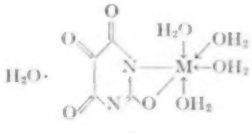
These results are in agreement with previous findings regarding the biological activities of metal chelates themselves in comparison with those of the chelating agents. Antitubercular activity, for instance, was observed *in vivo* with a series of chelated azo compounds (12), and the action of streptomycin was significantly extended when streptomycin chelates were employed in antibacterial tests (13). Albert (14) has also presented evidence that 8-hydroxyquinoline exerts antibacterial activity in the form of metal chelates, rather than by a possible chelation of metal.

CHELATE FORMATION

The preparation of the metal chelates was found to proceed in aqueous alkaline solution, similarly to the preparation of metal chelates of riboflavin (15). Aqueous solutions of metal salts were added to alloxan solutions in equimolar quantities at a *p*H of approximately 7.5, and the supernatant solution was brought to a *p*H of 5–6 to prevent conversion to alloxanic acid (7). The marked drop in *p*H which occurred in the absence of added alkali provides evidence for chelation rather than salt formation. Further evidence was provided by the absence of metal ion in the supernatant solutions as indicated by testing with dithizone and ferricyanide. This procedure gave immediate, colored precipitates with the divalent metals employed: namely, cobalt, nickel, and copper. The alloxanic acid chelate was obtained at a *p*H of 9. The necessity for further addition of alkali to maintain this *p*H again indicates chelate formation.

Other criteria for chelate formation listed by Martell and Calvin (16) and shown by the alloxan products include the following: colored products, decreased solubility in water, and elemental analyses of the isolated products which agree with the theoretical values. It was also found that decomposition of the chelate structures by acid did not take place in aqueous suspensions until a *p*H of 2 was reached. In addition, the colored products are not altered in appearance by dehydration at moderate temperatures, indicating the presence of water of crystallization.

The results of elemental analyses of the chelates, as shown in Table II, indicate a structure containing one metal atom per molecule of alloxan. The presence of water of chelation was shown by a loss of weight corresponding to one molecule of water when the chelates were dried *in vacuo* at 100°, and a loss corresponding to four more molecules of water by dehydration at 250°. The possibility of a polymeric structure having a 1:1 ratio of metal to alloxan is discounted by the presence of this amount of water of chelation. The following structure (I) can therefore be advanced as a reasonable one for a divalent, hexacoordinate metal chelate of alloxan.



I

TABLE I.—BLOOD SUGAR INCREASES CAUSED BY THE METAL CHELATES IN RABBITS

Metal Chelate	Administration	Dose, mg./Kg.	Increase in Blood Sugar Level, mg. %	Time, Hr.
Alloxan	i. p.	20	48	1
			4	2
Alloxan-Co	i. v.	20	35	3
Alloxan-Ni	i. p.	20	64	1
			42	2
Alloxanic acid-Co	i. p.	20	0	2
Riboflavin-Co[a]	i. p.	20	72	1
			−26	4
Isoalloxazine-Co[b]	i. v.	20	224	3
			0	6
Isoalloxazine[c]	i. p.	20	30	1
			−36	4

[a] The dicobalt chelate of riboflavin (15).
[b] The dicobalt chelate of 6,7-dichloro-9-(1-D-sorbityl)-isoalloxazine (15).
[c] 6,7-Dichloro-9-(1-D-sorbityl)-isoalloxazine (15).

A Karl Fischer determination for water in the cobalt chelate confirmed the presence of one molecule of titratable water in a structure of the molecular weight postulated in I. The remainder of the water content, which could be driven off by high temperature heating, was not removed from the molecule by Karl Fischer titration. Ultraviolet or visible absorption spectra could not be obtained for the alloxan chelates due to their insolubility.

Anal.—Calcd. for $C_4O_4N_2Co{\cdot}5H_2O$: $4H_2O$, 24.93. Found: H_2O, 23.89.

Determination of Blood Sugar Levels.—Normal blood sugar levels were determined in rabbits prior to injection of the chelates. After injection, blood samples were taken at intervals, and the sugar content was determined by the standard Folin-Wu method using a Bausch and Lomb "Spectronic 20" Spectrophotometer.

TABLE II.—ANALYSES OF THE ALLOXAN-METAL CHELATES

Formula[a]	Calculated, %				Found, %[b]			
	C	H	M"[c]	$1H_2O$	C	H	M"	$1H_2O$[d]
$C_4O_4N_2Co{\cdot}5H_2O$	16.62	3.49	20.39	6.23	16.31	3.76	20.81	6.78
$C_4O_4N_2Ni{\cdot}5H_2O$	16.64	3.40	...	6.24	16.20	3.25	...	6.51
$C_4O_4N_2Cu{\cdot}3H_2O$	18.62	2.32	...	6.97	18.33	2.56	...	7.21

[a] The chelates were dried *in vacuo* (2–3 mm.) at 25° before analysis for carbon and hydrogen.
[b] The carbon-hydrogen analyses were determined by Weiler and Strauss, Oxford, England. The metal analysis was conducted by a standard gravimetric procedure (17).
[c] M" = divalent metal.
[d] This quantity is the loss of weight on drying at 100° *in vacuo* (1–2 mm.) for four hours. A Karl Fischer determination of water in the cobalt chelate gave 6.12%.

EXPERIMENTAL

Preparation of the Metal Chelates.—To a solution of 1.4 Gm. (0.01 mole) of alloxan in 50 ml. of distilled water was added gradually with stirring 0.01 mole of metal salt in 25 ml. of water along with sufficient 10% sodium hydroxide solution to maintain a *p*H of 7.5. The salts used were $CoCl_2{\cdot}6H_2O$, $CuCl_2{\cdot}2H_2O$, and $Ni(NO_3)_2{\cdot}6H_2O$. In the reaction with cupric chloride, a small quantity of ethanol was added to facilitate filtration.

The *p*H was then lowered to 5 or 6, and the colored precipitates were allowed to settle for several hours. They were filtered, washed free of hydroxide ion with cold water, and dried in a vacuum desiccator over calcium chloride at room temperature. The copper chelate was green in color, the nickel chelate was light green, and the cobalt chelate was purple. None of the chelates showed signs of melting or decomposition below 300°. The following yields were obtained, based on the structures postulated: cobalt chelate, 69%; nickel chelate, 66%; and the copper chelate, 72%.

The alloxanic acid chelate was obtained by the same procedure, except that the *p*H was maintained at 9.

Anal.—Calcd. for $C_4H_2O_5N_2Co{\cdot}4H_2O$: C, 16.62; H, 3.49. Found: C, 16.68; H, 2.51.

Analysis of the Metal Chelates.—Qualitative analysis after sodium fusion indicated the presence of nitrogen but the absence of halogen. Testing the chelates with zinc-uranyl acetate reagent gave a negative test for sodium. Aqueous suspensions of the chelates were slightly acidic to pHydrion paper.

Loss of weight was observed on drying the compounds *in vacuo* (1–2 mm.) at 100° over magnesium perchlorate. The weight lost corresponded to slightly more than one molecule of water in a period of four hours. Loss of weight corresponding to approximately four molecules of water resulted after drying the previously dried cobalt chelate at 200–250° for four hours.

The chelates were injected in most cases by the intraperitoneal route because of their low solubility. Doses of the chelates below 20 mg./Kg. gave very small changes in blood sugar levels. A dose of the isoalloxazine-cobalt chelate of 50 mg./Kg. killed one rabbit two hours after intravenous injection, at which time the blood sugar was 170 mg. % above the control. The alloxan-cobalt chelate at a dose of 50 mg./Kg. given intravenously increased blood sugar 35 mg. % with a fall to 30 mg. % below the control value over a period of seven hours, while a 100 mg./Kg. dose increased blood sugar 50 mg. % with a gradual fall to normal during seven hours.

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Alkaloids of *Chelidonium majus*, L., Leaves and Stems I*

dl-Tetrahydrocoptisine

By F. J. BANDELIN and W. MALESH

The supraterranean parts of *Chelidonium majus* yielded 0.11 per cent of *dl*-tetrahydrocoptisine as the dominant alkaloid with smaller quantities of chelidonine and protopine. An extraction and isolation procedure for *dl*-tetrahydrocoptisine is presented and certain of its derivatives and their physical and chemical properties are discussed.

CHELIDONIUM MAJUS, L. (Family-Papaveraceae), sometimes known as celandine is a perennial herbaceous plant growing wild in both Europe and America. Formerly used in medicine it was official as the entire plant in the U. S. P. 1890.

Although sporadic investigations of the plants' constituents have been reported from time to time, these have been almost exclusively conducted on the underground portions of the plant. No recent systematic investigation utilizing modern techniques has appeared.

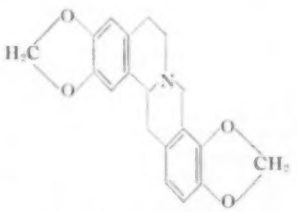
The reviews of Manske (1) and Henry (2) note that the underground portion of the plant contains alkaloids of the chelidonium and protopine subgroups. The former includes chelidonine, α-homochelidonine, methoxychelidonine, sanguinarine, and chelerythrine. The latter group comprises protopine and allocryptine. Sparteine, berberine, chelidonic acid, and coptisine have also been reported to be present.

This investigation concerns the alkaloids of the leaves and stems, and, in particular, the isolation and identification of *dl*-tetrahydrocoptisine, also known as *dl*-stylopine, hitherto unrecognized from this source. Since the completion of this work, however, the alkaloid has also been reported in this plant by Slavik (3).

Pharmacologically chelidonium has been used as a purgative in the form of a fluid extract prepared from the expressed juice (4). Mild narcotic and hypnotic properties (5, 6) have also been attributed to the plant. Hanzlik (7) has reported a nontoxic, morphine-like action for chelidonine observed by its action on smooth muscle.

The action of chelerythrine obtained from *Bocconia cordata* (8) has recently been shown to lower blood pressure in anesthetized cats, to initially stimulate the isolated small intestine and uterus of the rabbit and guinea pig, and to reduce the movements of the rat uterus. Thus only chelidonine and chelerythrine have been pharmacologically investigated and this only superficially.

dl-Tetrahydrocoptisine was first isolated by Manske (9) and has heretofore been found only in species of the genera *Corydalis*, *Dactylicapnos*, *Dicranostigma*, and *Fumaria* (10–12). Structurally *dl*-tetrahydrocoptisine is a bis-methylenedioxy isoquinoline derivative containing no methoxy or methylimino groups as in chelidonine and chelerythrine. It also lacks the hydroxyl group of coptisine at the 8-position



dl-Tetrahydrocoptisine

EXPERIMENTAL

The dried, whole supraterranean plant comprising the leaves and stems of *Chelidonium majus*[1] was ground to a medium fine powder in preparation for extraction.

Extraction of the Total Alkaloids.—In a typical run (Fig. 1) five pounds of the ground plant was moistened with 2 liters of concentrated ammonium hydroxide mixed with an equal volume of water, placed in a glass container and allowed to stand at room temperature for eighteen hours. The moist powdered plant was then dried in a circulating air oven at 40° for eighteen hours. When dry it was extracted with chloroform in an all-glass Soxhlet type extractor with three cycles of solvent and the chloroform extract was evaporated to dryness *in vacuo* yielding 90 Gm. (4.0%) of a black, wax-like material. This in turn was extracted with 6 one-liter portions of 5% acetic acid by heating each portion to 90° and permitting the mixture to cool

* Received May 16, 1956, from the Research Laboratories of Flint, Eaton and Company, Decatur, Ill.

[1] Obtained from the Meer Corporation, New York, N. Y.

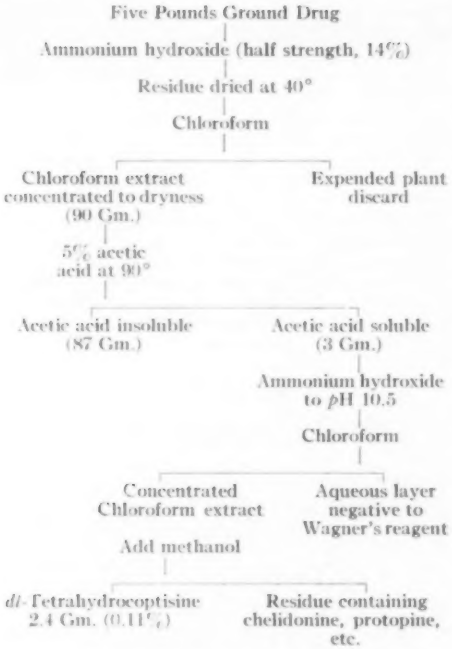

Five Pounds Ground Drug
Ammonium hydroxide (half strength, 14%)
Residue dried at 40°
Chloroform
Chloroform extract concentrated to dryness (90 Gm.)
Expended plant discard
5% acetic acid at 90°
Acetic acid insoluble (87 Gm.)
Acetic acid soluble (3 Gm.)
Ammonium hydroxide to pH 10.5
Chloroform
Concentrated Chloroform extract
Aqueous layer negative to Wagner's reagent
Add methanol
dl-Tetrahydrocoptisine 2.4 Gm. (0.11%)
Residue containing chelidonine, protopine, etc.

Fig. 1.—Schema for extraction of *Chelidonium majus* dried leaves and stems.

slowly to room temperature, decanting the acid extract and filtering.

The combined acetic acid extracts were made basic (*p*H 10) with concentrated ammonium hydroxide and extracted successively with 6 one-liter portions of chloroform. The combined solvent extracts were dried over anhydrous sodium sulfate and concentrated to a thick syrup (approximately 100 cc.) *in vacuo*. Upon the addition of 500 cc. of methanol, a gray crystalline precipitate appeared almost immediately. After eighteen hours at 5° the crystals were filtered, washed with cold methanol and dried to yield 2.4 Gm. (0.106%) of crude *dl*-tetrahydrocoptisine. The mother liquor, when evaporated to dryness *in vacuo*, yielded 0.6 Gm. of solid residue. Investigation of this residue by column chromatography on alumina revealed a slight additional amount of *dl*-tetrahydrocoptisine, chelidonine (0.002% of the plant), traces of protopine, homochelidonine, and an unidentified alkaloid obtained as an intensely yellow hydrochloride.

Purification of *dl*-Tetrahydrocoptisine.—Two grams of the crude dl-tetrahydrocoptisine was dissolved in 700 ml. of 5% aqueous acetic acid with the aid of heat and 150 ml. of concentrated hydrochloric acid was added. The alkaloid hydrochloride crystallized in the form of delicate white needles. This salt was recrystallized three times from methanol containing concentrated hydrochloric acid to yield sparkling white needles melting[2] at 266–269° with slight decomposition. The free base was extracted with chloroform and ammonium hydroxide and when recrystallized twice from a small amount of chloroform with ten volumes of methanol yielded 920 mg. of dl-tetrahydrocoptisine which melted sharply at 222–224°.

Derivatives and Analysis of *dl*-Tetrahydrocoptisine Base.[3]—The base purified as described, dried at 100° *in vacuo* for six hours and subjected to analysis agrees with the empirical formula $C_{19}H_{17}O_4N$.

Anal.—Calcd. for $C_{19}H_{17}O_4N$: C, 70.57; H, 5.29; N, 4.33. Found: C, 70.64; H, 5.07; N, 4.56.

Hydrochloride.—Prepared as previously described, dried at 100° *in vacuo* for six hours. *Anal.*—Calcd. for $C_{19}H_{17}O_4N \cdot HCl$: C, 63.42; H, 5.04; N, 3.89; Cl, 9.85. Found: C, 63.64; H, 4.80; N, 3.99; Cl, 10.08.

Picrate.—To 150 mg. of base dissolved in 100 ml. of 25% aqueous acetic acid, picric acid T. S. was added until no further precipitate appeared. After centrifuging and decanting the supernatent layer, the wet precipitate was dissolved in 100 ml. of ethanol by heating and filtering. On cooling, very fine lemon yellow needles appeared which on recrystallization from ethanol melted at 138–141° without decomposition. *Anal.*—Calcd. for $C_{19}H_{17}O_4N \cdot C_6H_3N_3O_7$: C, 54.34; H, 3.64; N, 10.14. Found: C, 53.57; H, 3.73; N, 9.71.

Since this assay was not in good agreement with the calculated percentages, the picrate was recrystallized from ethanol. This derivative melted sharply at 138–139° without decomposition. It was dried at 84° *in vacuo* for eight hours and upon analysis yielded the following results: C, 53.11; H, 3.68; and N, 9.82. The analytical results indicate some degree of decomposition occurring possibly during drying.

Nitrate.—To a solution of the base in acetic acid, ammonium nitrate was added. On standing tiny hemispherical needle clusters or very small needles of *dl*-tetrahydrocoptisine nitrate formed. These were recrystallized from ethanol. The nitrate did not melt distinctly but decomposed gradually up to 230°.

Nitrite.—On the addition of sodium nitrite to a solution of the alkaloid in aqueous acetic acid, nitrous oxide was evolved and *dl*-tetrahydrocoptisine nitrite crystallized on standing. The latter melted at 233° with decomposition.

The base could not be acetylated and it was found to be optically inactive. It is soluble in chloroform and in glacial acetic acid and slightly soluble in hot methanol, ethanol, isopropanol, benzene, and 5% acetic acid solution. The base may form an adjunct with acetone since recrystallization from this solvent caused a considerable lowering of the melting point. A chloroform solution of the alkaloid discolors (yellow) in a few days in the daylight at room temperature.

The Labat reaction (12) for methylenedioxy groups is positive; the color deepening from a clear green to blue on heating in a water bath.

The ultraviolet absorption spectrum was determined by using a Beckman Quartz Spectrophotom-

[2] All melting points were determined with Fisher-Johns apparatus and are uncorrected.

[3] All analyses by the Clark Microanalytical Laboratories, Urbana, Ill.

eter—Model DU. Maxima were found at 250, 290, and at 355 mμ; minima were found at 235, 260, and at 330 mμ (Fig 2).

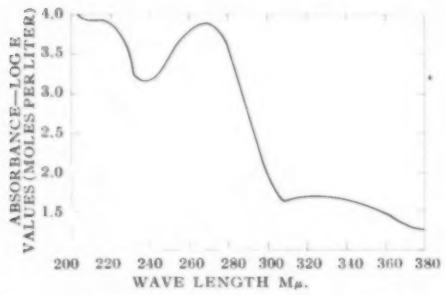


Fig. 2.—Molar extinction coefficient of *dl*-tetrahydrocoptisine in ethanol.

Paper Chromatography

Using the ascending technique of Levine and Fishbach (14) and paper moistened with phosphate-citrate buffer at *p*H 3.5 and using *n*-butyl acetate, *n*-butanol and formic acid, 25:5:1, gave the following R_f values when sprayed with bromophenol blue in 90% ethanol: *dl*-tetrahydrocoptisine, 0.87; chelidonine, 0.34; protopine, 0.27.

The systems *n*-butanol, acetic acid and water always gave unsatisfactory results yielding elongated spots without resolution.

Color Reactions of *dl*-Tetrahydrocoptisine with Various Reagents.—Acetic-sulfuric acid, colorless to lavender in ten minutes, dark red in one hour; Frohdes reagent, green at first, then yellow to blue; persulfate, deep green through aquamarine and green to yellow and orange; Marquis reagent, blue-green to wine red; nitric acid, concentrated, yellow to orange.

l-Tetrahydrocoptisine was first isolated by Schlotterbeck and Watkins (15) from *Stylophorum diphyllum*. The *d*- form was later isolated by Späth and Julian (16). The latter on oxidation with iodine in alcohol is converted into a yellow quaternary iodide which can be reduced to *dl*-tetrahydrocoptisine. The *dl*- base may be resolved with *d*- bromo-camphor-π-sulfonic acid in dilute acetic acid from which the *l*- base is separated by crystallization (m. p. 203–204°). From the mother liquors the *d*- base may be separated (m. p. 203–204°) by crystallization as the tartrate.

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An "o-Hydroquinone" Analog of Thyroxine*

By H. R. WETHERELL

The synthesis of a new type of derivative of thyroxine is presented. This "o-hydroquinone" analog was prepared in order to explore further the hypothesis that a quinoid-like equilibrium is important in determining thyroxine-like action. The analog possessed some thyromimetic activity as shown by biological assay, but was not sufficiently active to justify the unequivocal conclusion that the quinoid-like equilibrium is essential.

SINCE THE synthesis of thyroxine by Harington and Barger (1), many congeners of this hormone have been prepared. The demonstrated physiological activity of certain of these compounds coupled with the inactivity of others, have led to postulations concerning the "obligatory requirements" for thyroxine-like activity (2). Selenkow and Asper (3) have recently reviewed this topic and have summarized the apparent requirements. The evidence now available appears to justify the following postulates: a diaryl ether or diaryl sulfide structure, an oxygen substituent at the 2′ or 4′ position of the second phenyl ring, substitution of an electronegative group (halogen, nitro, etc.) in at least the 3 position on the first phenyl nucleus, and an activating side chain on position 1 of the first phenyl nucleus seem to be essential for activity

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In 1941 Niemann and his associates synthesized two isomers of thyroxine, one possessing thyroxine-like activity and one without activity (4, 5). They then proposed a theory of thyroxine action based on an assumed equilibrium between thyroxine and a quinoid form of thyroxine as shown in Fig. 1. The isomer of thyroxine

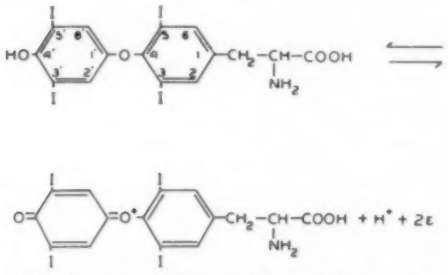

Fig. 1.—Quinoid equilibrium reaction involving thyroxine. The numbering system of the thyronine nucleus is also indicated.

that would permit formation of this hypothetical quinoid form (with the hydroxyl group in the 2′ position) was physiologically active, whereas the isomer which would not permit quinoid formation (with the hydroxyl group in the 3′ position) was inactive. In a later review, however, (6) Niemann mentioned that the proposed equilibrium has not as yet been shown to occur.

The recent demonstrations of Gross and Pitt-Rivers of the occurrence of 3,5,3′-triiodothyronine[1] in thyroid tissue (7) and in plasma (8), have stimulated interest in other compounds that have fewer than four iodine atoms attached to the thyronine nucleus. According to Roche and his co-workers (9, 10), 3,3′,5′-triiodothyronine and 3,3′-diiodothyronine are also present in hydrolysates of thyroglobulin. The triiodothyronine isolated by Gross and Pitt-Rivers has been shown to be from five to ten times as active as thyroxine, while the triiodothyronine of Roche and his group possesses almost as much activity as thyroxine. The diiodothyronine however, has only one-tenth the activity of thyroxine. It should be mentioned here that the relative potency of analogs of thyroxine depends to a large extent upon the assay method chosen, but the "antigoitrogenic" method devised by Dempsey and Astwood (11) is the one most frequently used now.

[1] The name thyronine is commonly used to indicate the deiodinated thyroxine residue. Chemically it is 4-(4′-hydroxyphenoxy)-DL-phenylalanine or DL-α-amino-β-(4-(4′-hydroxyphenoxy)-phenyl)propionic acid. The naturally-occurring iodinated thyronines are members of the L series of amino acids.

A consideration of Niemann's "quinoid hypothesis" and the fact that certain thyroxine analogs bearing only two or three iodine substitutions are quite active, led the author to synthesize and assay the compound to be reported here, 3,5-diiodo-3′-hydroxythyronine. The substance can be viewed as an *o*-hydroquinone analog of thyroxine in which the 3′ iodine atom has been substituted by an hydroxyl group and the 5′ position bears a hydrogen atom in place of the fourth iodine atom. Quinone formation could take place with ease, and if this particular kind of equilibrium is important, then this compound would be expected to exhibit thyromimetic activity. Figure 2 shows the compound prepared and the presumed equilibrium.

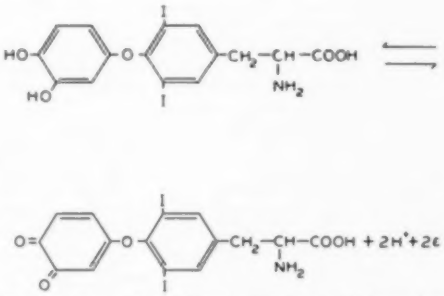

Fig. 2.—The *ortho*-hydroquinone analog of thyroxine and its quinoid form.

EXPERIMENTAL

The experimental procedures employed were patterned after the syntheses reported by Niemann and his co-workers, hence only a brief reference to some of the intermediate products will be made.

4-Nitroveratrole.—Veratrole (Eastman Kodak Company) was nitrated by the method of Cardwell and Robinson (12) to give 4-nitroveratrole in essentially quantitative yield, m. p. 95–96°.[2]

4-Aminoveratrole.—This was prepared by reducing 4-nitroveratrole with sodium sulfide according to the method of Fargher (13). The crude amine could be diazotized directly but considerable tar resulted. Yields of the corresponding hydroxy compound were improved by using amine that had been purified by distillation *in vacuo*, b. p. 165–168° (18 mm.), followed by recrystallization from ether. A 75% yield of light tan product melting at 87–88° was obtained.

4-Hydroxyveratrole.—Diazotization of the above amine was carried out according to the method of Baker and Evans (14). After recrystallization from carbon tetrachloride a 40% yield of 4-hydroxyveratrole was obtained, m. p. 80–81°.

3,4,5-Triiodonitrobenzene.—This compound was prepared by the method of Niemann and Redemann (4) in 85% yield, m. p. 165–166°.

[2] All melting points are corrected.

3,5-Diido-4-(3′,4′-dimethoxyphenoxy)-nitrobenzene.—A mixture of 40 Gm. of 4-hydroxyveratrole, 86 Gm. of 3,4,5-triiodonitrobenzene, 50 Gm. of finely powdered freshly dehydrated potassium carbonate and 170 cc. of freshly distilled 2-pentanone was refluxed in an oil bath for six hours. Water was added to dissolve the salts and the volatile products were removed by steam distillation. The dark brown crystalline residue, after cooling, was recovered, washed with water and cold methanol, and dried. Recrystallization from 2-butanone gave 57 Gm. of bright yellow needles, m. p. 168.5° to 169.5°. The yield was 63% of the theoretical.

Anal.—Calcd. for $C_{14}H_{11}O_5NI_2$: N, 2.66; I, 48.3. Found: N, 2.60; I, 49.0.

The demethylated derivative was prepared by refluxing a small sample of the dimethoxy compound in a mixture of 2 cc. of acetic anhydride and 2 cc. of hydriodic acid (d. 1.7) for one hour. Upon cooling, solid material separated out; this was filtered off, washed with a little cold acetic anhydride and recrystallized from ethanol-water giving pale yellow needles, m. p. 203–204°.

Anal.—Calcd. for $C_{12}H_7O_5NI_2$: N, 2.81; I, 50.9. Found: N, 2.95; I, 51.3.

The dibenzoate was prepared by reacting a sample of the *o*-hydroquinone with an excess of benzoyl chloride at 100° for one hour. The reaction mixture was poured into an excess of cold 5% aqueous sodium hydroxide and allowed to stand until the unreacted benzoyl chloride had hydrolyzed. The precipitate was filtered off, washed thoroughly with water, and recrystallized from ethanol-water. The resultant colorless prisms had m. p. 166–167°.

Anal.—Calcd. for $C_{26}H_{17}O_7NI_2$: N, 1.98; I, 35.9. Found: N, 2.10; I, 35.2.

3,5-Diiodo-4-(3′,4′-dimethoxyphenoxy)-aniline.—To a hot solution of 55 Gm. of the nitro compound in 220 cc. glacial acetic acid kept at 95–100° on a steam bath, was added 75 Gm. of stannous chloride dihydrate. The solution was saturated with dry hydrogen chloride gas at 95–100°. After two hours the double salt of the amine with stannic chloride separated out. The mixture was cooled and the precipitate filtered off and sucked dry. The salt was decomposed with an excess of cold 50% aqueous sodium hydroxide, the temperature being maintained below 10°. The resultant paste was extracted with ten 200-cc. portions of ether. The ether solution was dried over anhydrous sodium sulfate and the solvent removed by distillation at atmospheric pressure. The amine was left as a light tan residue which could be purified by recrystallization from ethanol-water giving colorless needles, m. p. 162.5° to 163.5°.

Anal.—Calcd. for $C_{14}H_{13}O_3NI_2$: N, 2.82; I, 51.1. Found: N, 2.90; I, 51.5.

The acetyl derivative prepared in the usual manner and recrystallized from ethanol formed pale pink needles, m. p. 176–177°.

Anal.—Calcd. for $C_{16}H_{15}O_4NI_2$: N, 2.60; I, 47.1. Found: N, 2.71; I, 47.5.

The hydrochloride of the amine was prepared by passing dry hydrogen chloride gas into a solution of the amine in ether. The salt prepared in this manner had a m. p. 191–192° (with decompn). It could not be recrystallized however, since in every attempt the compound lost a molecule of hydrogen chloride and reverted to the free amine, m. p. 162–163°. The salt as prepared was pure enough for the subsequent reaction.

3,5-Diiodo-4-(3′,4′-dimethoxyphenoxy)-benzonitrile.—Twenty-five grams of 3,5-diiodo-4-(3′,4′-dimethoxyphenoxy)-aniline hydrochloride was suspended in 260 cc. of glacial acetic acid containing 15 cc. of water. To this suspension was added slowly 6 Gm. of freshly distilled *n*-butyl nitrite. After standing for fifteen minutes the diazonium solution was added slowly to a stirred potassium cuprocyanide solution prepared by adding a solution of 160 Gm. of potassium cyanide in 260 cc. of water to a solution of 140 Gm. of cupric sulfate pentahydrate in 520 cc. of water. The temperature was maintained below 10° during the addition. The reaction mixture was then warmed to 80°, cooled, and the solid collected. After thorough drying the solid was extracted with five 100-cc. portions of boiling benzene. The dark brown benzene solution was concentrated *in vacuo* to a volume of 100 cc. and passed through a column of activated alumina. The column was eluted with a mixture of benzene and acetone (90:10, by volume) and the dark orange eluate taken to dryness on the steam bath. The red-orange residue was recrystallized with the aid of Norit® from acetone-water giving pale pink plates, m. p. 185–186°. The yield was 10 Gm. (40%).

Anal.—Calcd. for $C_{15}H_{11}O_3NI_2$: N, 2.76; I, 50.1. Found: N, 2.85; I, 51.0.

3,5-Diiodo-4-(3′,4′-dimethoxyphenoxy)-benzaldehyde.—Twenty grams of anhydrous stannous chloride was suspended in 110 cc. of anhydrous ether and dry hydrogen chloride gas was passed into the suspension at 0° until a single phase had been formed. To this solution was added a solution of 8.2 Gm. of the nitrile dissolved in 50 cc. of anhydrous chloroform. Considerable hydrogen chloride was given off and when this dissolution ceased the flask was stoppered and allowed to stand at room temperature. After two hours a heavy yellow oil had separated and after twenty-four hours this oil had changed to a crystalline mass. The dark yellow solid was filtered off, washed with a little cold ether and suspended in 50 cc. of 6 *N* hydrochloric acid. The aldimine double salt hydrolyzed rapidly as the hydrochloric acid suspension was brought to a boil. After cooling, the pale yellow solid was filtered off and dried. One recrystallization from ethanol-water gave 3.8 Gm. of colorless plates, m. p. 169.5° to 171°.

Anal.—Calcd. for $C_{15}H_{12}O_4I_2$: I, 49.8. Found: I, 50.1.

4-(3′,5′-Diiodo-4′-(3″,4″-dimethoxyphenoxy)-benzal)-2-phenyloxazolone-5.—A mixture of 2 Gm. of the aldehyde, 2 Gm. of anhydrous sodium acetate, 0.75 Gm. of hippuric acid, and 25 cc. of freshly distilled acetic anhydride was heated on a boiling water bath for thirty minutes. During the course of heating the mixture turned light orange and a yellow precipitate settled out. The reaction mixture was cooled and poured into 200 cc. of cold water and allowed to stand until the acetic anhydride had hydrolyzed. The solid was collected by filtration and dried. The material crystallized from cellosolve-water (80:20, by volume) as lemon-yellow plates, m. p. 204–205°, yield 1.6 Gm.

Anal.—Calcd. for $C_{24}H_{17}O_5NI_2$: N, 2.14; I, 38.9. Found: N, 2.20; I, 39.3.

DL-3,5-diiodo-4-(3′,4′-dihydroxyphenoxy)phenylalanine.—A mixture of 8 cc. of acetic anhydride, 8 cc. of hydriodic acid (d. 1.7), 1 Gm. of red phosphorus, and 1 Gm. of the azlactone was refluxed under nitrogen for one hour. The phosphorus was filtered from the hot solution and washed with two small portions of hot glacial acetic acid. The filtrate was evaporated to dryness *in vacuo* in a stream of nitrogen, the residue treated with a little hot water saturated with nitrogen and evaporated again to dryness *in vacuo*. The residue was decolorized with a dilute aqueous solution of sulfur dioxide and the resultant pale yellow solid collected and dried, care being taken to exclude oxygen during these operations. The solid was boiled with 2 *N* hydrochloric acid, some insoluble material filtered off, and the hot filtrate adjusted to *p*H 5 with 10 *N* ammonium hydroxide. Upon cooling, the amino acid separated as a semicrystalline pale pink solid. The material could be recrystallized only with difficulty and well formed crystals could not be obtained. Oxidation by atmospheric oxygen presumably was responsible for the slight coloration of the material. When heated in a melting point block the amino acid turned brown at about 180°, iodine was evolved and decomposition took place at 220–221°. The yield was 0.2 Gm.

Anal.—Calcd. for $C_{15}H_{13}O_5NI_2$: N, 2.59; I, 47.0. Found: N, 2.40; I, 46.1.

The amino acid gave a color reaction with ninhydrin and when dissolved in dilute alkali a purple solution resulted. Decomposition with formation of colored products upon treatment with alkali is characteristic of most catechol derivatives.

Attempts to iodinate this amino acid in the catechol portion of the molecule resulted in extensive decomposition and no reaction products could be isolated. Iodine monochloride and iodine in a potassium iodide solution were used unsuccessfully as iodinating agents. Fukamauchi (15) has reported negative results in his attempts to iodinate catechol derivatives by a number of different methods.

TABLE I.—RESULTS OF BIOASSAY

Group[a]	Treatment	Thyroid Weight[b]
A	Controls—solvent alone	20.4 ± 1.42
B	3 μg. of L-thyroxine[c]	14.3 ± 1.08
C	6 μg. of L-thyroxine[c]	10.4 ± 0.76
D	100 μg. of analog[c]	17.2 ± 0.69

[a] Six rats per group.
[b] The mean, expressed as mg./100 Gm. of body weight ± S. E.
[c] Per rat.

Bioassay.—The assay employed was the "goiter-prevention" method, and was carried out essentially as reported by Gross and Pitt-Rivers (16). It is based on the ability of thyroxine and thyromimetic substances to inhibit the development of goiter brought about by the administration of thiouracil. Table I summarizes the results of this assay. The rats (male sex, initial weight 80–100 Gm., Sprague-Dawley strain) were housed two to a cage and given drinking water containing 3% dextrose and 0.1% thiouracil *ad libitum*. For ten days each animal was given a daily subcutaneous injection of either the solvent or drug dissolved in the solvent; the volume injected in every case was 0.1 cc. The solvent was 0.8% sodium chloride made 0.002 *N* with sodium hydroxide; each 0.1 cc. contained 50 μg. of sodium bisulfite to prevent oxidation. On the eleventh day the animals were weighed, sacrificed, and the thyroids removed and weighed.

RESULTS AND DISCUSSION

The data presented in Table I indicate that the *o*-hydroquinone analog has an activity about 1/60 that of L-thyroxine in the goiter prevention assay. The values in column three are all statistically significantly different from each other, and when a log dose-response curve is plotted from the data for L-thyroxine a value for the potency of the analog can be obtained.

It was thought that had the analog reported here possessed considerable thyroxine-like activity a clue would have been given to a possible mechanism of action of thyromimetic substances, namely participation in some important cellular redox system; Niemann's hypothesis would also have been strengthened. A potency relative to L-thyroxine of 1/60 admittedly is not strong evidence along these lines, but it is suggestive, and although the assay method used was chosen because it measured a representative action of thyroxine-like materials, the possibility exists that in other biological systems the analog might exhibit a greater activity. Other considerations are the likelihood that the compound was not able to penetrate into cells and that due to the labile nature of the catechol portion of the molecule it was oxidized and destroyed too rapidly. It would be expected that the addition of the sodium bisulfite would minimize the latter, however.

SUMMARY

1. DL-3, 5-diiodo-4-(3′,4′-dihydroxyphenoxy)-phenylalanine, an analog of thyroxine, has been synthesized.

2. The bioassay of the analog by the "anti-goitrogenic" method in rats showed it to be 1/60 as active as L-thyroxine.

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The Assay of *Rauwolfia serpentina* Preparations*

By DANIEL BANES, JACOB WOLFF, HERMAN O. FALLSCHEER, and JONAS CAROL

A method is proposed for the colorimetric assay of *Rauwolfia serpentina* root and its preparations, based upon the reaction between reserpine-rescinnamine group alkaloids and nitrous acid.

IN A PREVIOUS publication from this laboratory (1) we have described a procedure for the chemical evaluation of *Rauwolfia serpentina* preparations. In that procedure, the feebly basic fraction containing reserpine and rescinnamine was isolated by column chromatography, the alkamine esters were saponified, and the derived trimethoxycinnamic and trimethoxybenzoic acids were determined spectrophotometrically. The procedure recommended here for the routine assay of *Rauwolfia serpentina* preparations employs classical methods for extracting the mixed alkaloids, and a modified Szalkowski-Mader nitrite test (2) for their determination.[1]

Other methoxyindole alkaloids which occur in *Rauwolfia serpentina* root, including reserpinine and methyl reserpate, yield fluorescent products similar to those of reserpine and rescinnamine in the nitrite test. These known interferences are eliminated in the extraction with acid, as shown by paper chromatography on the extracts, as well as by recovery experiments on pure alkaloids. But small quantities of other unidentified methoxyindole alkaloids may persist. For this reason, the substances determined colorimetrically in the proposed method of assay are designated as "reserpine-rescinnamine group alkaloids." The large blanks encountered are probably due to oxidation derivatives of reserpine and rescinnamine, which occur naturally in the plant (1), and are extracted with the active alkaloids.

PROPOSED METHOD OF ASSAY

Materials

Soxhlet Extraction Apparatus.—A medium-sized extractor provided with a 250-ml. flask and a 35 x 80 mm. thimble is most convenient, although a smaller apparatus may be used.

Solvents.—Ethanol, chloroform of U. S. P. or A. C. S. grade. 1,1,1-trichloroethane: Redistill in all-glass apparatus, and collect the fraction boiling at 73–76°.

Solutions.—(*a*) Reserpine standard solution: Dissolve 20.0 mg. of crystalline reserpine standard in 25 ml. of hot ethanol, cool, and dilute to exactly 50 ml. with ethanol. When stored in a tightly stoppered, light-resistant bottle in the dark, this solution is chromogenically stable for several weeks. Dilute 5.0 ml. to 100 ml. with ethanol. The diluted reserpine standard solution contains 20 mcg. reserpine per ml. (*b*) Sulfuric acid solution 0.5*N*: Dissolve about 30 ml. of sulfuric acid in two liters of water. (*c*) Aqueous sodium bicarbonate solution, 2%. (*d*) Aqueous sodium nitrite solution, 0.3%. (*e*) Aqueous sulfamic acid solution, 5%. Prepare fresh every two or three days.

Boiling Chips.—Silicon carbide grains, 20 mesh.

PROCEDURE

Extract 2–3 Gm. of finely powdered *Rauwolfia serpentina* root or the equivalent in powdered tablets in a Soxhlet extraction apparatus for four hours. Use about 100 ml. of vigorously boiling ethanol as solvent, and a few boiling chips to prevent bumping. Protect the flask and thimble, and all solutions of Rauwolfia alkaloids from direct or strong light.

Wash the extract into a 100-ml. volumetric flask with ethanol, cool, dilute to the mark, and mix. Transfer a 20-ml. aliquot to a separator containing 200 ml. of sulfuric acid solution, mix, and extract with three 25-ml. portions of trichloroethane. Drain the lower phase as completely as possible. Wash each of the trichloroethane extracts in a second separator containing 50 ml. of the sulfuric acid solution, and discard.

Extract the weakly basic alkaloids from the aqueous solution with 25, 15, 15, 10, 10, and 10 ml. of chloroform. Wash each chloroform extract with the acid in the second separator, then with two 10-ml. portions of sodium bicarbonate solution in two additional separators. Filter the chloroform extracts through cotton into a 100-ml. volumetric flask containing 10 ml. of ethanol. Dilute to exactly 100 ml., and mix.

Transfer duplicate 10.0-ml. aliquots to 150 x 18 mm. test tubes and mix with 4 ml. of ethanol. Add a few boiling chips, and heat to boiling in a water bath below 70°. Raise the bath temperature continuously, and heat until ebullition in the tubes *just* ceases (avoid prolonged heating in the absence of solvent). Wipe the outsides of the warm tubes, place in a vacuum desiccator, and evaporate to dryness. Dissolve the residues by agitating with 5.0 ml. of ethanol. Transfer duplicate 5-ml. aliquots of the diluted Reserpine standard solution (20 mcg./ml.) to test tubes. Add 2.0 ml. of sulfuric acid solution to one of the sample tubes and to one of the standard tubes (the blanks). Add to the other tubes 1.0 ml. of sulfuric acid solution and 1.0 ml. of sodium nitrite solution. Mix the contents

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[1] We are indebted to Jack Cooper of Ciba Pharmaceutical Products, Inc., Summit, N. J., for calling this color test to our attention, and to the authors for permitting us to study the manuscript prior to publication.

of each tube, and warm in a water bath at 50–60° for twenty minutes. Cool, add to each tube 0.5 ml. of sulfamic acid solution, and mix. After stabilization of the solutions, determine their absorbances in matched 1-cm. cuvettes at 390 mμ relative to a (2+1) ethanol-water mixture. The quantity of reserpine-rescinnamine group alkaloids, in mg., in the sample weighed is given by the formula: $[(A - A_0)/(S - S_0)] \times 5$, where A and A_0 are the absorbances of the nitrite-treated sample and sample blank, respectively, and S and S_0 are the corresponding absorbances for the standard solution aliquots.

DISCUSSION

To check the accuracy of the proposed procedure, 2.5 mg. of reserpine was heated with 100 ml. of boiling alcohol in a Soxhlet extractor for four hours and then treated as described for *Rauwolfia serpentina* preparations. Recovery of reserpine indicated a loss of not more than 1%. When 15 Gm. of the exhausted marc of several samples was analyzed according to the proposed method, chromogens equivalent to less than 0.3 mg. of reserpine were recovered, or less than 1% of the reserpine-rescinnamine group alkaloids in an average root sample.

A comparison of data obtained by applying the chromatographic-spectrophotometric procedure and the nitrite procedure to twenty samples of powdered roots and tablets is presented in Table I. Except for Sample 9, the nitrite procedure yielded identical or somewhat higher values for all of the root samples. The data show a good correlation between procedures. By both methods of analysis, roots 1, 2, and 3 possess the highest concentrations of active alkaloids, and 9 and 10 the least. On the basis of numerical criteria derived from these data (150–250 mcg./100 mg. of authentic root) tablet samples 11, 12, 13, 16, and 17 would be deemed acceptable by both procedures and samples 14, 15, and 19 would be considered below standard by both procedures. Discrepancies arise in the values for samples 18 and 20, both borderline cases.

The nitrite procedure is more convenient and more reproducible than the chromatographic procedure. Furthermore, it is less vulnerable to the interfering effects of dyes and emulsifiers which may be incorporated in commercial tablets. It does not differentiate between reserpine and rescinnamine, and would require an accompanying paper chromatogram to indicate the distribution of these alkaloids. However, such chromatograms are desirable in any case for the identification of the root species (1). It is felt that the nitrite procedure supplemented by a paper chromatogram would provide a suitable method for the chemical assay of *Rauwolfia serpentina* preparations.

TABLE I.—ASSAYS OF RAUWOLFIA SERPENTINA PREPARATIONS

Sample	Description	Chromatographic Reserpine-Rescinnamine	Nitrite Reserpine-Rescinnamine Group Alkaloids,
		—Mcg./100 Mg. or— Mcg./Tablet	
1	Powdered Root	245	249
2	Powdered Root	238	246
3	Powdered Root	220	241
4	Powdered Root	216	237
5	Powdered Root	204	217
6	Powdered Root	200	232
7	Powdered Root	186	184
8	Powdered Root	184	184
9	Powdered Root	166	158
10	Powdered Root	157	174
11	Tablet, 100 mg.	185	201
12	Tablet, 100 mg.	182	186
13	Tablet, 100 mg.	157	149
14	Tablet, 100 mg.	136	140
15	Tablet, 100 mg.	133	141
16	Tablet, 50 mg.	116	124
17	Tablet, 50 mg.	82	77
18	Tablet, 50 mg.	78	69
19	Tablet, 50 mg.	72	72
20	Tablet, 50 mg.	72	82

CONCLUSIONS

A modified nitrite procedure has been proposed for the assay of *Rauwolfia serpentina* preparations. Data obtained by the use of this procedure show a good correlation with those obtained by chromatographic analysis.

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The Oxidative Degradation of Reserpine Preparations*

By DANIEL BANES, JACOB WOLFF, HERMAN O. FALLSCHEER, and JONAS CAROL

Reserpine elixirs and injections may undergo partial oxidative degradation. A modified nitrite procedure is employed for the determination of reserpine in the presence of its decomposition products.

THE SUSCEPTIBILITY of reserpine to various types of decomposition has been reported by several investigators (1–3). In alkaline media the diester may be hydrolyzed to trimethoxybenzoic acid and methyl reserpate or reserpic acid. When exposed to light, solutions of reserpine in organic solvents, such as chloroform, acquire a yellow color with green fluorescence. Dechene (2) has observed that reserpine in acidic aqueous solution is slowly oxidized to a fluorescent substance at room temperature.

The ready conversion of reserpine to fluorescent derivatives has been utilized for the estimation of the alkaloid. Dechene (2) accelerated the oxidation by heating with hydrogen peroxide. McMullen and his co-workers (3) heated reserpine with sulfuric acid, and measured both the fluorescence and the ultraviolet absorption of the yellow products. In the procedure recommended by Szalkowski and Mader (4) for elixirs, injections, and tablets, reserpine was treated with nitrous acid at room temperature, and the absorption of the fluorescent product was determined at 390 mμ. Of these procedures the nitrite treatment yielded the most reproducible results in this laboratory. But none of them provides for the possible conversion of reserpine to oxidative degradation products during the fabrication or storage of its preparations.

We have reported previously (5) that highly fluorescent collutes were isolated from several reserpine preparations by column chromatography. Acidified alcoholic solutions of these collutes exhibited maxima near 387 and 266 mμ, and a minimum near 315 mμ. Thus, the spectra obtained resemble those published by McMullen, *et al.* (3), and by Szalkowski and Mader (4) for their fluorescent derivatives. Weisenborn and Diassi (6) have reported similar data for pure 3-dehydroreserpine nitrate, with a relatively higher absorption maximum at 387 mμ. The chief product in the oxidation of reserpine with hydrogen peroxide in glacial acetic acid yielded an ultraviolet absorption spectrum similar to that of 3-dehydroreserpine nitrate in acidified alcoholic solution. In alcoholic solution basified with ammonia, the peak at 387 mμ shifted and exhibited a doublet with maxima at 318 and 331 mμ. Weisenborn (7) has shown that this hypsochromic shift with bifurcation is typical of 3-dehydroreserpates. The spectra of the fluorescent collutes recovered from chromatographic columns showed an identical shift. These oxidative degradation products of reserpine therefore may be characterized as 3-dehydroreserpates.

It is apparent that 3-dehydroreserpates would be estimated as reserpine in the colorimetric and fluorimetric methods cited, unless correctives were applied. In a modified nitrite procedure for the assay of *Rauwolfia serpentina* preparations (8) a blank determination was included to correct for substances absorbing light at 390 mμ prior to nitrite treatment. The reaction was hastened by moderate application of heat, and excess nitrous acid was destroyed by the addition of sulfamic acid to yield stable fluorescent solutions. Otherwise, the procedure was essentially that described by Szalkowski and Mader (4), and is applicable to reserpine preparations.

EXPERIMENTAL

Preparation of Samples

Tablets.—A quantity of powdered tablets containing about 5 mg. of reserpine was mixed with 40 ml. of ethanol and heated to simmering for twenty minutes. The mixture was filtered through a loose cotton pledget into a 100-ml. volumetric flask. The solids and filter were washed thoroughly with several portions of hot ethanol. The cooled filtrate was diluted to volume, and mixed. A 20-ml. aliquot was mixed with 200 ml. of acid, and analyzed as previously directed for *Rauwolfia serpentina* preparations (8).

Elixirs and Injections.—A volume of solution equivalent to 1 mg. of reserpine was transferred to a separator, mixed with sufficient ethanol to yield a total alcohol volume of 20 ml. and analyzed as directed (8).

DISCUSSION

Ethanol solutions contining 1.00 mg. of crystalline reserpine were extracted as directed for the samples,

* Received August 25, 1956, from the Division of Pharmaceutical Chemistry, U. S. Food and Drug Administration Department of Health, Education, and Welfare, Washington 25, D. C.

and compared with unextracted standards. At least 97% of the alkaloid was recovered, with no discernible production of 3-dehydroreserpates. The columns of data in Tables I and II headed "Uncorrected % Reserpine" were calculated by direct comparison of A_{280} measurements on samples and extracted standards. "Corrected % Reserpine" values were based on the same measurements corrected for the blanks.

TABLE I.—ANALYSIS OF RESERPINE PREPARATIONS BY THE NITRITE PROCEDURE

Sample	Description and Declaration	Reserpine, % of Declaration	
		Corrected	Uncorrected
1	Injection, 2.5 mg./ml.	90.0	97.6
2	Injection, 2.5 mg./ml.	87.6	94.4
3	Injection, 2.5 mg./ml.	96.4	99.2
4a	Injection, 2.5 mg./ml.	88.8	98.0
4b	Injection, 2.5 mg./ml.	90.0	99.1
5	Injection, 2.5 mg./ml.	94.8	98.0
6	Elixir, 0.25 mg./4 ml.	92.4	108.3
7	Elixir, 0.25 mg./5 ml.	99.4	108.2
8	Elixir, 0.25 mg./5 ml.	84.8	115.1
9	Elixir, 0.25 mg./5 ml.	102.4	118.0
10	Elixir, 0.25 mg./5 ml.	104.0	118.0
11	Tablet, 0.25 mg.	110.3	120.4
12	Tablet, 0.1 mg.	91.8	92.7
13	Tablet, 2.0 mg.	95.0	96.2
14	Tablet, 0.25 mg.	99.2	99.3
15	Tablet, 1.0 mg.	95.5	96.4
16	Tablet, 0.1 mg.	96.5	97.0
17	Tablet, 0.25 mg.	95.2	96.0
18	Tablet, 0.5 mg.	90.8	91.6
19a	Tablet mixture, 0.128%	99.3	99.3
19b	Tablet mixture, 0.128%	100.0	100.4
20	Lactose triturate, 1%	97.5	97.5

Results obtained by applying the modified nitrite procedure to reserpine tablets, injections, and elixirs are shown in Table I. Comparison of the corrected and uncorrected values for injections and elixirs demonstrates the necessity for the blank correction. Every injection sample exhibited a green fluorescence. Similarly, the first chloroform extract of every elixir fluoresced markedly. Considering Dechene's findings, degradation of the alkaloid in these preparations might have been anticipated since they are essentially acidic aqueous solutions of reserpine.

Most of the commercial tablet samples examined in this laboratory have been free of oxidation products. In Sample 19, prepared from purified reserpine by dry mixing to simulate a tablet mixture, no oxidation product was detected, and recoveries were practically quantitative. However, decomposition may be encountered occasionally even in tablets, as in the case of Sample 11.

TABLE II.—COMPARISON OF NITRITE AND CHROMATOGRAPHIC PROCEDURES

Sample	Declaration, %			
	Nitrite Corrected for Blank	Chromatographic Reserpine	Nitrite Uncorrected	Chromatographic Total Alkamines
1	90.0	90.8	97.6	98.4
2	87.6	88.4	94.4	94.8
3	96.4	99.6	99.2	100.8
6	92.4	95.2	108.3	106.8
7	99.4	99.0	108.2	111.0
11	110.3	113.2	120.4	119.0

Several of the samples reported in Table I were also analyzed by column chromatography (5). A comparison of data obtained by the two methods is presented in Table II. The corrected values in the modified nitrite procedure are within 3% of the assay values for reserpine isolated chromatographically. Furthermore, the uncorrected nitrite values are in excellent agreement with the figures for total alkamines in the chromatographic procedure, comprised of reserpine plus the fluorescent degradation product calculated as reserpine. These data indicate that the modified nitrite method may be employed to determine the quantity of intact reserpine in its pharmaceutical preparations.

CONCLUSIONS

It has been shown that reserpine elixirs and injections are susceptible to oxidative degradation. A modified nitrite procedure has been employed for the determination of reserpine in the presence of its degradation products. This procedure yields assay data in accord with those obtained by chromatographic analysis.

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Book Notices

Introductory Organic Chemistry. 3rd ed. By E. WERTHEIM and HAROLD JESKEY. McGraw-Hill Book Company, Inc., New York, 1956. vii + 476 pp. 16 x 23.5 cm. Price $5.50.

This is a general text on organic chemistry intended to acquaint the student with the subject, and it does not attempt to go beyond fundamental concepts. The treatment is clear and as simplified as possible. Teachers whose students are not science majors will find this book helpful. Study questions are given at the end of each chapter. A glossary of chemical, biological, and medical terms, a list of reference books, and a subject index are appended.

Organic Chemistry. 3rd ed. By LOUIS F. FIESER and MARY FIESER. Reinhold Publishing Corporation, New York, 1956. v + 1112 pp. 15.5 x 24 cm. Price $10.

This book attempts to develop the basic principles and concepts of modern organic chemistry and then to relate them to technology, and to biochemistry and medicine. Many new developments since the 1949 (2nd) edition have been included in the extensively rewritten 3rd edition. The molecular orbital theory, mechanisms of ionic and free radical reactions, and resonance are introduced early in the text and referred to throughout.

Concepts of conformational analysis are developed and used in discussions of sugars, terpenes, alkaloids, and steroids. Structures of reserpine, cyanocobalamin, and the total synthesis of aldosterone are included. The text includes 454 brief biographical references to chemists associated with material cited. The final chapter is devoted to chemotherapy.

An aid to the teacher and student is the inclusion of problems at the end of some chapters and the inclusion of the answers at the end of the book. The format and printing are excellent. An author index and a subject index are appended.

This book should interest all teachers of organic chemistry and particularly those who teach in pharmacy and premedical schools. It is an excellent reference book for all chemists.

1956 Medical Progress. A Review of Medical Advances During 1955. MORRIS FISHBEIN, Editor. McGraw-Hill Book Company, Inc., New York, 1956. x + 389 pp. 15.5 x 24 cm.

This is the fourth volume in this series which reviews in a concise and interesting manner the many advances in medicine during the preceding year. The review of the 1955 volume appeared in THIS JOURNAL, **44,** 456(1955). Subjects covered by different authors in the 1956 volume are: medicine, allergy, rheumatic diseases, diabetes, gynecology, gastroenterology, laboratory procedures, general surgery, ophthalmology, poliomyelitis vaccine, neurology, physical medicine and rehabilitation, dermatology, nutrition, cardiovascular disease, psychiatry, neuropsychiatry, new drugs, endocrinology, ear, nose and throat, and orthopedic surgery. Dr. Paul L. Wermer, in his introduction to the chapter on New Drugs, states: "Probably the most important event of the year in relation to new drugs was the emergence of the ataraxic (ataractic) agents as a new class of medicaments." Classed as ataraxics are rauwolfia, reserpine, chlorpromazine (Thorazine), and Frenquel (α-4-piperidyl diphenyl carbinol HCl). The drugs meprobamate (Miltown, Equanil), and mephenesin "probably are more properly considered as nervous system depressants." While production of prednisone and prednisolone represent an improvement over earlier corticoid compounds because of reduced severity and incidence of side effects, "the ideal glucocorticoid substance without mineralocorticoid effect has not been synthesized as yet." Any medical or pharmaceutical reference shelf would be more valuable with this series of books included.

BOOKS RECEIVED

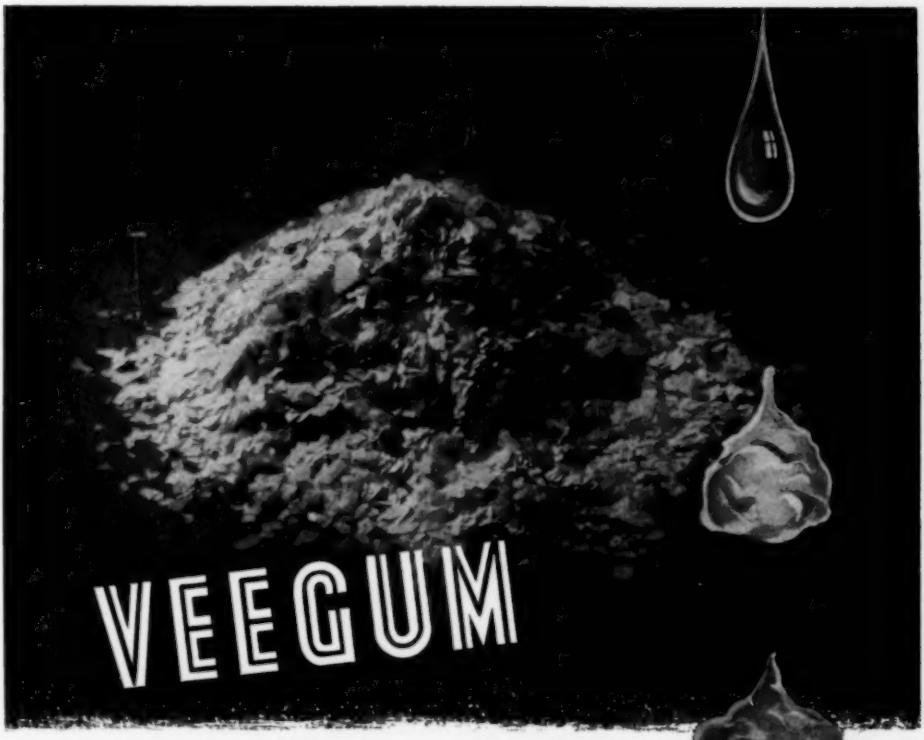
Biology of the Laboratory Mouse. Edited by GEORGE D. SNELL. Dover Publications, Inc., New York, 1956 (Reprint of first edition, 1941). viii + 497 pp. 16 x 24.5 cm. Price $6.

Catalysis. Vol. 4. By PAUL H. EMMETT. Reinhold Publishing Corporation, New York, 1956. vi + 570 pp. 15.5 x 23.5 cm. Price $12.50.

Pharmaceutical Index. 1st ed. The Japan Pharmaceutical Association, Tokyo 1956. 695 pp. 13 x 18.5 cm.

Travaux des Laboratoires de Matière Médicale et de Pharmacie Galénique de la Faculté de Pharmacie de Paris. Vol. XL. (In four parts). By M. M. JANOT and R. PARIS. Vigot Frères, Paris, 1956. 15.5 x 24 cm.

Observations on Krebiozen in the Management of Cancer. By A. C. IVY, JOHN F. PICK, and W. F. P. PHILLIPS. Henry Regnery Company, Chicago, 1956. 88 pp. 14.5 x 22 cm. Price $2.50.



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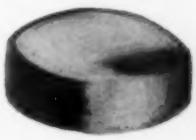

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